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Penicillin in the Treatment of Peritonitis Due to Liver Autolysis in Dogs.
(17734)

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Since the observation of Mann(1) that small pieces of dogs' liver left free within the peritoneal cavity would cause death within 16 hours was confirmed and extended by Mason and Davidson(2), interest in this phenomenon has generally centered about explanations of the lethal outcome. Debate has arisen on the question of whether anaerobic bacteria normally present in the dogs' liver play a decisive or merely a secondary role in producing the fatal toxemia of this condition. It is thought that the experiments to be described will help to answer this question and also demonstrate a successful treatment for what has previously been described as a uniformly fatal condition.

Experiments of Mason and Davidson were repeated by Wangenstein and Waldron(3)

and by Haden and Orr(4) with similar results. Ellis and Dragstedt(5) first suggested the importance of bacteria normally present in the dogs' liver in producing a fatal infection in the dead liver. They called attention to the earlier work of Wolbach and Saiki(6) who had described an anaerobic, spore-forming, gas-producing bacillus found in normal dogs' liver. They supported their thesis by confirming the presence of this bacillus in dogs' liver and by showing that liver sterilized by autoclaving, or sterile liver taken from fetal dogs produced no toxic effects in the peritoneal cavity of dogs. Disagreement arose when Andrews and Hrdina(7) reported that

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3. Wangenstein, O. H., and Waldron, G. W., *Arch. Surg.*, 1928, v17, 430.

the toxic reaction of autolytic peritonitis could be provoked by the implantation of finely ground sterile liver; they described the toxic agent as probably an albumose arising from liver autolysis. They felt that the bacterial invasion was of secondary importance, probably produced by an increased permeability of the bowel, allowing bacteria to pass through and then infect the material lying within the peritoneal cavity. Trusler, Reeves and Martin(8) who, after careful bacteriological studies, came to disagree with Andrews and Hrdina, found that fresh ground liver sterilized by autoclaving would not cause death when injected intraperitoneally unless it was contaminated.

Methods and results. The 25 healthy, dewormed dogs used in these experiments were divided into 3 groups. The animals in Groups I and II were subjected to the intraperitoneal autolysis of segments of their own liver. The dogs in Group I had penicillin treatment and those in Group II did not; otherwise their treatment was similar. Under intravenous pentobarbital anesthesia and using aseptic precautions, the abdomen was opened and the quadrate lobe of the liver, weighing from 4.5 g to 34.0 g and averaging 17.6 g, was removed and dropped into the free peritoneal cavity. Bleeding from the liver was controlled with mattress sutures. Among the animals of Group I penicillin treatment was begun from 1 to 4 hours postoperatively, when each dog received an intramuscular injection of 300,000 units of penicillin G in oil and wax.* This dose of penicillin was repeated daily for a total of 4 to 11 injections in the dogs that recovered. Of the dogs in the penicillin-treated group only 3 of the 12 died while all of the 5 control animals died within $3\frac{1}{2}$ days or less following surgery. With one exception, the penicillin-treated animals which died all survived longer than any of the control animals. At autopsy there was a characteristic picture of peritoneal injection, brownish serosanguinous fluid and gas in the peritoneal cavity, and frequently a spongy pale

remnant of the free liver segment still in the peritoneal cavity. Microscopic sections of the necrotic free segments of liver showed many rod-shaped bacteria. None of the survivors had any evidence of peritonitis at autopsy. In several of them necrotic remnants of liver were found encapsulated by omentum.

The dogs in Group III were exposed to the intraperitoneal autolysis of fresh segments of human liver from 11.0 g to 44.0 g and averaging 30.8 g in weight. Specimens of human liver were obtained aseptically from recently dead cadavers with no intra-abdominal disease and placed in the peritoneal cavity of dogs through a wound made aseptically under intravenous pentobarbital anesthesia. Only 2 of the 7 non-penicillin-treated dogs exposed to human liver autolysis died. Neither of the animals dying of human liver autolysis showed the large amount of serosanguinous intraperitoneal fluid characteristic of canine liver autolysis.

Discussion. The short survival of the control animals with devitalized canine liver is the typical result of this experiment. There has been only one dog described in the literature(9) who has survived more than 5 days after a small, free, non-autoclaved segment of adult dog liver has been placed in the peritoneal cavity. This regularly fatal outcome has followed even when attempts have been made to treat the dogs by gas gangrene antitoxin(10), by correcting their anhydremia(11), or by abdominal drainage(11).

The results of these experiments argue strongly for the importance of bacteria in the mechanism of death due to intraperitoneal canine liver autolysis and seem to support the theory of Ellis and Dragstedt(5) that infection originating in the devitalized liver is the cause of death. In the recent brief report of Markowitz, Rappaport, and Scott(12), it

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12. Markowitz, J., Rappaport, A., and Scott, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, v70, 305.

* Provided by E. R. Squibb and Sons, New York City.

8. Trusler, H. M., Reeves, J. R., and Martin, H. E., *Arch. Surg.*, 1935, v30, 371.

is stated that dogs with totally ligated hepatic arteries usually survived when given massive doses of penicillin, whereas the control animals usually died. These findings also emphasize the importance of bacteria in causing death due to liver autolysis in dogs.

To our knowledge the relatively benign effect of placing segments of human liver in the peritoneal cavity of dogs has not been previously described. The fact that devitalized human liver is less toxic than devitalized dog liver may be due to the absence in the human liver of the anaerobic, spore-forming, gas-producing organism found normally in dogs' liver. No such organism was found in the human liver specimens used in this experiment, though *Aerobacter aerogenes* and *staphylococci* were found. Shann and Frad-

kin(13) describe a patient who recovered following sequestration of a large fragment of liver and review the literature describing several other cases of recovery following liver sequestration secondary to biliary tract surgery or abdominal trauma. These clinical findings seem to be in agreement with our observation as to the relative non-toxicity of the devitalized human liver as compared with dog liver.

Conclusions. 1. Treatment with penicillin in oil reduces the fatality of peritonitis due to canine liver autolysis in dogs. 2. Autolysis of devitalized human liver in dogs is less fatal than autolysis of canine liver in dogs.

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Hormonal Mechanisms in Nervous Mechanism of Gastric Acid Secretion in Humans.* (17735)

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It has been shown that the so-called chemically induced secretion of gastric acid occurring in response to the ingestion of food depends upon the elaboration of an histamine-like hormone, which has been named gastrin(1-8). Moreover, the pyloric end of the

stomach has been identified as the source of this hormone(1-8), although hydrochloric acid secreting parietal cells are known to be generally distributed throughout the whole stomach(9). Recently several workers have adduced evidence that the secretion of gastric acid in response to vagal impulses also requires the mediation of histamine (9-12) or the histamine-like hormone gastrin(13-15). As a by-

* Aided by grants from the Commonwealth Fund and the Estate of Lester N. Hofheimer.

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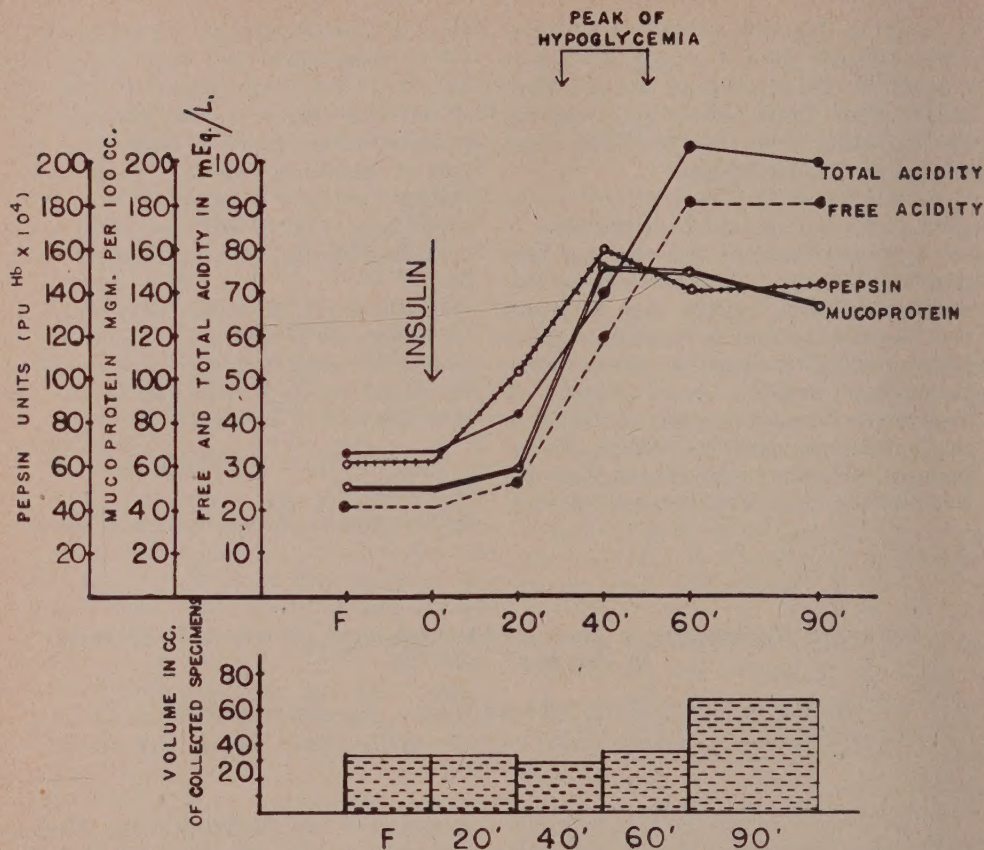


FIG. 1.

Average response of gastric secretion to I.V. injection of 16 U. insulin in eight cases of gastro-duodenal ulcer.

product of recent studies of the secretory activities of the gastric glands(16-18) new light has been cast upon this question.

Material and methods. Studies were carried out on 4 subjects with normal stomachs, 8 with duodenal ulcer and 7 subjects in whom subtotal gastric resection had been performed for peptic ulcer. After 2 fasting specimens of gastric juice had been withdrawn through a Levine tube at intervals of 20 minutes, 16 units of regular insulin was administered to each subject intravenously. Subsequently gastric specimens were aspirated at 20, 40, 60 and

90 minutes after injection. In each specimen acidity was determined by the usual titration technic with Toepfer's reagent and phenolphthalein. Pepsin concentration was measured by the photometric modification of the hemoglobin method(19) and gastric mucoprotein by the colorimetric tyrosine methods of Glass and Boyd(20).

Observations. A. Normal subjects and those with duodenal ulcer. In gastric juice from the intact stomach, whether or not duodenal ulcer was present, there was observed uniformly a rise in the concentration of all three secretory products of gastric glands, namely acid, pep-

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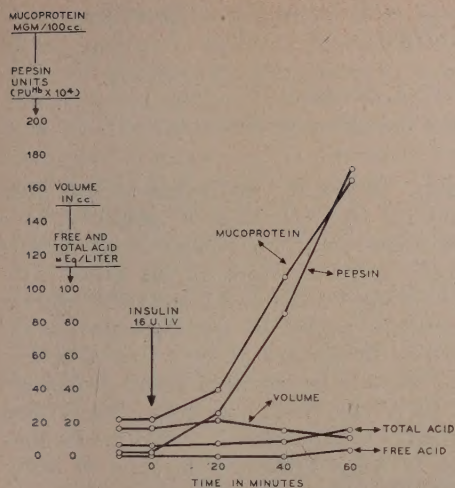


FIG. 2.

Average response of gastric secretion to I.V. injection of 16 U. of insulin in seven subjects, following removal of the distal $\frac{2}{3}$ to $\frac{3}{4}$ of the stomach for peptic ulcer.

sin and mucoprotein following intravenous injection of insulin. The full data are reported elsewhere (16,17). Fig. 1 illustrates graphically the average changes. It will be noted that the peak of elevation of pepsin and mucoprotein coincided with the symptoms of hypoglycemia (pallor, sweating, weakness and hunger) and that the peak of acid response was delayed 20-40 minutes. A moderate elevation of acid, however, was present coincidental with the peak of pepsin and mucoprotein.

Comment. Numerous workers have shown that the lowest levels of blood sugar occur 20-30 minutes after intravenous administration of insulin (21-23). The above data indicate that the elevation of pepsin and mucoprotein correspond much more closely in point of time to the hypoglycemic reaction than does the rise in acid concentration. This suggests the possibility of an intermediary mechanism being involved in the latter reaction although some direct effect is suggested

by the fact that moderate elevation of acid often coincided with the peak of pepsin and mucoprotein concentration.

B. Observations following partial gastric resection. Surgical removal of the distal $\frac{2}{3}$ - $\frac{3}{4}$ of the stomach for peptic ulcer altered the response of these 3 constituents to insulin administration. While elevation of pepsin and mucoprotein still occurred following insulin injection, a significant increase in acid and volume of secretion occurred in only 1 out of the 7 subjects. Fig. 2 illustrates graphically the average changes observed. Full data on this dissociated response by the gastric glands are presented elsewhere.

Comment. It has been shown that parietal cells are generally distributed throughout the body of the stomach (9,24,25). Thus the failure in most cases of insulin to induce an increased concentration of acid when only the antral end of the stomach has been removed, while elevation of pepsin and mucoprotein continue to occur, requires special explanation. The data suggest that the gastrin containing pyloric end of the stomach is involved in the acid secreting mechanism which is set in motion by vagus stimuli.

Conclusion. 1. The peak of elevation of gastric acidity in the intact stomach following insulin injection occurs significantly later than that of pepsin and mucoprotein, and 30-45 minutes after the maximal fall in blood sugar.

2. The acid stimulating effect of insulin is diminished and often abolished following resection of the distal end of the stomach while the pepsin and mucoprotein response are undiminished.

3. These observations add further strength to already existing evidence that while vagal stimuli appear to affect directly the glandular cells which secrete pepsin and mucoprotein and may act in part directly on the parietal cells, they also effect an increased elaboration of HCl in the human stomach by the intermediation of another mechanism that may involve the hormone "gastrin".

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A Rough Antigen Frequently Associated with Vi in Some Enterobacteriaceae. (17736)

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Felix and Pitt(1) first described the Vi antigen as a thermolabile antigen conferring virulence and O-inagglutinability upon organisms possessing this component. Many investigators(2,3,4) have questioned the thermolability of the Vi antigen, others(5,6) its role in virulence and it is well known that an occasional Vi positive strain is completely O-agglutinable in pure O antiserum. Stuart, Wheeler *et al.*(7) found that certain coliform and *Paracolobactrum* cultures gave a characteristic Vi type of agglutination in a presumably pure Vi antiserum. Adsorption of the Vi antiserum with any one of the coliform or *Paracolobactrum* cultures completely removed agglutinins for all these cultures without reducing the titer for Vi positive *Salmonella* strains while adsorption with certain Vi positive *S. typhosa* and *S. ballerup* strains removed all agglutinins. The antigen common to some coliform, *Paracolobactrum* and Vi positive *Salmonella* cultures but differing from the Felix and Pitt antigen was called a Vi-like component. Wheeler and Stuart(8) found the Vi-like antigen in one culture with part of and 2 cultures with the complete somatic structure of Sach's Q-771 strain. Subsequently the same antigen was found in several additional strains of coliform, *Paracolobactrum* and Shigella-like organisms (unpublished

data). Brower (9) reported an antigen other than the IX, XII, Vi or d component in a Bhatnagar strain of *S. typhosa* as a "probably rough antigen". Work on the Vi-like substances was revived in this laboratory when a nonmotile variant of the Bhatnagar strain used by Brower and kindly furnished by her was found to be composed almost exclusively of the Vi and Vi-like antigens. The Vi-like antigen will hereinafter be called the Er antigen (a rough antigenic component of many Enterobacteriaceae).

In the present work pure Vi antisera were prepared in two ways. (1) Antisera prepared in rabbits with living antigens of strains of *S. typhosa* possessing the Vi but lacking the Er antigen were adsorbed with living antigens of H-901 and O-901. (2) antisera of rabbits immunized with alcoholic extracts of *S. ballerup* containing Vi produced for the most part only Vi antibodies. An occasional antiserum prepared from an alcoholic extract of any Vi positive organism, particularly after prolonged immunization, possessed antibodies reacting in low dilutions (20-160) with boiled homologous antigen. Pure Er antiserum was prepared with *S. typhosa* O-484 (the S-107 strain of Stuart, Wheeler *et al.*(7) containing both the Vi and Er antigens. This antiserum after adsorption with *S. typhosa* possessing Vi but not Er yielded a pure Er antiserum. Another Er antiserum was made from *Paracolobactrum coliforme* 121 rich in Er but lacking the Vi, IX, XII or d antigen. Tests with cultures known to possess the Vi or Er antigen or both indicated that the Er antibodies prepared from the *S. typhosa* and *P. coliforme* strains were identical. The Er antiserum prepared from *P. coliforme* 121 agglutinated *S. typhosa* O-484 to a titer of 20480 and other *S. typhosa* strains possessing the Er antigen to titers ranging from 80 to 20480. With these antisera the Er antigen

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was found in high concentration in 10 strains of *S. typhosa*, in moderate concentration in 6 strains and in weak concentration in several others from a total of 75 strains studied. It was found in *S. ballerup* strains, in one rough *paratyphi* C strain, in 4 *Paracolobactrum* strains and 2 *Escherichia coli* cultures. These findings would indicate that the Er antigen is relatively widespread in the Enterobacteriaceae. The confusion that can be caused by the presence of Er antibodies in presumably pure Vi antiserum is apparent. The increasing number of reports of Vi antigen in coliform and *Paracolobactrum* cultures must be viewed with suspicion unless confirmed by adequate adsorption tests. If for no reason other than to avoid confusion with Vi the general distribution of the Er antigen should be recognized.

In the previous work(7) it was noted that coliform and *Paracolobactrum* cultures with Er antigen exhibited rough tendencies. When antigens of these strains were prepared by adding saline to agar slant cultures and the tubes rotated, the growth came off the agar in flakes. When briskly rotated for 2 or 3 minutes most of the flakes broke up and after allowing the suspension to sediment 10 to 15 minutes perfectly smooth antigens that showed no spontaneous agglutination in 0.85 or 2% saline at 37°C or 55°C were obtained. Upon boiling for 1 or more hours in 0.85% saline varying degrees of spontaneous agglutination did occur with all strains. Young colonies of these cultures on nutrient agar plates showed rough characteristics which disappeared as the age of the colony increased. In broth cultures sediment was noted on the bottom of the tube but smooth, stable antigens could be pipetted from the supernatant fluid. *S. typhosa* strains with Er antigen reacted in a similar manner except that the tendency toward roughness was not usually as marked as with coliform and *Paracolobactrum* cultures. If the Er antigen is a rough component of a culture a comparison of the Vi and Er antigens with respect to distribution and other characteristics is important. The Er antigen has been found in all groups of enteric bacteria possessing Vi: *Salmonella*, coliform and *Para-*

colobactrum(10). In the *Salmonella*, including *S. typhosa*, the Er antigen has been limited to those cultures possessing Vi, but not all strains with Vi possess the Er antigen. In the coliform and *Paracolobactrum* the Er antigen alone is usually found. Hirsch(11) found that cultures with Vi were agglutinated by acriflavin. Cultures containing Er antigen showed somewhat stronger agglutination than Vi strains in the same dilution or agglutinated in slightly higher dilutions of the dye than did the Vi strain. Agglutination in acriflavin long has been accepted as one of the better though not infallible criteria of roughness. Both the Vi and Er antigens may be extracted from cultures by alcohol and by saline and heat; the former more readily by alcohol and the latter by saline and heat. These extracts are antigenic in rabbits and will inhibit the agglutination of the Vi antigen or the Er antigen by their respective antibodies (Stuart and Kennedy)(12). White(13) and others have pointed out that treatment of rough *Salmonella* with alcohol may remove the rough character of the culture. When cultures with the Vi or Er antigen are heated to 60°C for one hour most or all of these antigens are freed from the cell and the washed cells are not agglutinated in Vi or Er antisera respectively. Free in the supernatant fluid the Vi and Er antigens are not completely destroyed for an hour or more at 121°C. The Er antigen is not as readily dissociated from the cell as the Vi and in some strains may resist dissociation for one hour at 60°C. Increasing the time of heating at 60°C or increases in the temperature dissociates the Er antigen but may cause the residual cells to be spontaneously agglutinated. Cultures containing both Vi and Er antigens tested in pure Er antiserum often exhibited Er-inagglutinability comparable to O-inag-

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glutinability usually associated with the Vi antigens(14). One difference may be noted between the reaction of Vi and Er antigens in their respective antisera. With the former the agglutination which occurs after 4 hours at 37°C is frequently completely reversed after several hours at 55°C. The Er antigen which tends to agglutinate weakly at 37°C may agglutinate more strongly in some antisera at 55°C.

In *S. typhosa* the principal characteristic of rough strains appears to be the complete or almost complete absence of the IX, XII somatic antigens. All rough strains examined in this laboratory to date and meeting this requirement have possessed both the Vi and Er antigens. Agglutination and adsorption tests with an antiserum prepared from "*S. typhi* 6S. rough" showed that the Vi and Er antigens were the only major somatic components of this strain. Saline suspensions of agar slant cultures of this strain while exhibiting no spontaneous agglutination after 2 hours at 37°C and 18 hours at 55°C were completely agglutinated after one hour in a boiling water bath. Saline suspensions of *P. coliforme* 121 which possessed a strong Er but no Vi antigen behaved in the same manner at 37°C, 55°C and 100°C. However, *P. coliforme* 121 completely lost the Er antigen when after numerous platings an extremely rough variant was established.

Finally the presence of the Vi and Er antigens in an organism is definitely associated with the opaque colonial form while their absence is associated with the translucent colony. Arkwright(15) early in his work on the smooth to rough transformation pointed out the tendency of the smooth colony variants

to be translucent and the rough to be opaque. With both the Vi and Er antigens the association with colonial form is not absolute and exceptions do occur. Whether the acquisition of the Vi antigen is a preliminary stage in the smooth to rough transformation with the acquisition of the Er antigen a definite step in that direction cannot be answered at this time. In any event the Er antigen is important in itself from a diagnostic viewpoint since strains of many enteric bacteria containing the Er antigen may be reported as Vi positive. As previously pointed out(16) so called "single factor" or "pure typing serums" lend a false sense of security to diagnostic bacteriology and too much reliance is often placed on agglutination tests in such serums. A simple adsorption test usually will differentiate between the Vi and Er antigens but the Er antigen may vary both quantitatively and qualitatively in different cultures and even in the same culture upon different occasions.

Specific designation (Er) is given to the antigen under discussion only because of its frequent association with the Vi antigen in enteric bacteria, particularly the *Salmonella* group.

Conclusions. 1. In some Enterobacteriaceae, particularly *S. typhosa*, a rough antigen (Er) has been found associated with Vi. 2. As with Vi, heat and/or alcohol dissociates the Er component from the cell and the free antigen can be used to produce Er antibodies in rabbits or to inhibit agglutination of cultures possessing this antigen by Er antiserum. 3. Like Vi the presence of the Er antigen is associated with the opaque colonial form while its absence is associated with the translucent form.

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Synthesis and Microbiological Testing of 1,1-Aminopropanesulfonic Acid.* (17737)

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McIlwain(1) has shown that the 1,1-aminosulfonic acid analogues of glycine, alanine, valine, and leucine inhibit the growth of several bacteria. In some cases the inhibition was reduced only by the corresponding aminocarboxylic acid; in others, however, the inhibition was reduced by other amino acids as well. It was the purpose of these investigations to synthesize 1,1-aminopropane sulfonic acid, analogous to the aminocarboxylic acid, α -aminobutyric acid, which does not occur in nature, and to test the sulfonic acid for its inhibitory or growth-promoting effects in a bacterial system.

Synthesis. 1,1-aminopropanesulfonic acid was synthesized by the addition of 50% aqueous ammonium bisulfite solution to a five per cent excess of propanal. The volume of the mixture was reduced under vacuum to one quarter the original volume. Ethyl alcohol was added till precipitation began and the mixture was placed in the refrigerator for crystallization. The crystals collected were purified by redissolving them in a minimum of water, adding ethyl alcohol, and again crystallizing in the cold.

$C_3H_9O_3NS$. Calculated, C 25.90, N 10.06, S 23.04; Found, C 26.65, N 9.97, S 23.33.

Microbiological method. The organism used for testing was *Lactobacillus casei* carried in tomato agar slabs. For the experiments, a 2% dextrose, 2% yeast extract broth was used as the basal medium. For a given test, 5 ml of basal medium were added to each test

TABLE I.
Effect of 1,1-Aminopropanesulfonic Acid on Growth of *Lactobacillus casei*.

1,1-aminopropane-sulfonic acid, M	Growth (turbidity)
0	103
1×10^{-5}	103
5×10^{-5}	102
1×10^{-4}	94
$2 \times "$	92
$4 \times "$	90
$6 \times "$	82
$8 \times "$	67
1×10^{-3}	57
$2 \times "$	42
$4 \times "$	28
$6 \times "$	20
$8 \times "$	10
1×10^{-2}	0

tube in a series, the aminocarboxylic acids and water were added. The tubes were then sterilized by autoclaving at 15 lb pressure for 15 minutes. A solution of the sulfonic acid, sterilized by filtration through a Seitz filter, was added to the autoclaved tubes after they were cooled. The final volume of each tube was 10 ml. The tubes were inoculated with *Lactobacillus casei* and incubated at 37°C for 24 hours; the growth was determined by turbidimetric measurements.

Results. The 1,1-aminopropanesulfonic acid began to inhibit growth at a concentration of 1×10^{-4} M; complete inhibition was obtained at 1×10^{-2} (Table I). Alpha-aminobutyric acid did not reverse the inhibition produced by the sulfonic acid analogue, but instead inhibited the growth still more. This result is not surprising since Gladstone(2) has shown that α -aminobutyric acid is toxic to microorganisms. In view of the investigations showing the overlapping growth-reversal effect produced by aminocarboxylic acids of aminosulfonic acid inhibition(1), it might be expected that the inhibition produced by 1,1-

* Contribution No. 251 from the Department of Biochemistry and Nutrition, University of Southern California.

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2. Gladstone, G. P., *Brit. J. Exp. Path.*, 1939, v20, 189.

aminopropanesulfonic acid could be reversed by such natural amino acids as glycine, alanine, valine, or the leucines. None of these amino acids, however, produced an increase in growth at the concentrations used. In addition, simple mixtures of natural amino acids were ineffective in reversing the inhibition. The addition of from one to three milliliters of a solution of sixteen amino acids[†] to the basal medium in the presence of the aminosulfonic acid produced about a 2% increase in growth. However, addition of the same amino

[†] The amino acid mixture contained in each ml, 0.8 mg each of DL-leucine, DL-serine, DL-phenylalanine, DL-glutamic acid, DL-valine, DL-aspartic acid, L-cystine, DL-arginine hydrochloride, L-tryptophane, L-tyrosine, DL-threonine, DL-methionine, DL-alanine, DL-isoleucine, DL-lysine and L-histidine hydrochloride.

acid mixture to the basal medium in the absence of the aminosulfonic acid resulted in a similar increase in growth. This would indicate that the increased growth was due to a general improvement in the nutritive value of the medium rather than a reversal of inhibition by a specific amino acid.

Further investigations will be necessary to determine whether or not inhibition produced by 1,1-aminopropanesulfonic acid is reversed by substrates other than amino acids.

Summary. 1,1-aminopropanesulfonic acid was synthesized by the addition of ammonium bisulfite to propanal. The aminosulfonic acid inhibited the growth of *Lactobacillus casei* at levels from 10^{-4} to 10^{-2} molar. Single amino acids as well as combinations of amino acids were ineffective in relieving the inhibition.

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Growth of *Mycobacterium tuberculosis* in Egg Yolk Mediums. (17738)

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Egg yolk is widely used as the chief ingredient in a variety of solid mediums for the cultivation of *Mycobacterium tuberculosis*; in fact, no other nutrients need to be added(1). That it can be equally useful in liquid and semi-liquid mediums is not so well known. Besredka(2) described a liquid medium made by autoclaving egg yolk diluted with alkaline water; this preparation, however, does not support the growth of small inocula. Tubercle bacilli will grow well within the yolks of market eggs(3) even from inocula as small as 10 tubercle bacilli(4), as well as in the yolks of embryonated eggs(5). The wide-

spread use of the Dubos Tween-albumen mediums has demonstrated the value of liquid mediums in metabolic and chemotherapeutic studies of the tubercle bacillus. The measure of the adequacy of such mediums is their ability to promote rapid growth from small inocula (10 organisms or less). Several easily prepared mediums that will do this can be made from egg yolk.

Methods. Strictly fresh chicken eggs were used. The yolks were removed aseptically and diluted with sterile water, salt solutions, or a basal medium made up according to a formula given by Dubos *et al.*(5):

1. Corper, H. J., and Cohn, M. L., *Am. Rev. Tuberc.*, 1942, v46, 560.
2. Besredka, A., *Ann. de l'Inst. Pasteur*, 1921, v35, 291.
3. Gelarie, A. J., *J. Lab. Clin. Med.*, 1944, v29, 532.
4. Eggerth, A. H., Drescher, E., and McOsker, V. C., *Am. Rev. Tuberc.*, 1948, v57, 632.

Casein digest (N-Z Amine)* or asparagine	g 2.0
Na ₂ HPO ₄ · 12H ₂ O	6.3
KH ₂ PO ₄	1.0
Na citrate · 2H ₂ O	1.5
Dissolve, then add	
Ferrie ammonium citrate	0.1
MgSO ₄ · 7H ₂ O	0.6
Distilled water to 1000 ml. Adjust to pH 6.8.	

*N-Z Amine (Type B), Sheffield Farms Co., Inc.

TABLE I.
Growth of *Mycobacterium tuberculosis* in Egg Yolk Mediums.

Type of medium	Dilution of yolk	Growth in days									
		12	14	16	18	20	22	25	28	31	35
Autoclaved in distilled water	1-10	1+	4+								
	1-50		1+				4+				
	1-100				1+			4+			
	1-300					no growth					
Autoclaved in basal medium	1-10	1+	4+								
	1-50			1+					4+		
	1-250						1+			4+	
	1-1000							1+			4+
	1-2500								1+		2+
	1 5000					no growth					
Unheated yolk in basal medium	1-10	1+	4+								
	1-100	1+	4+								
	1-1000		1+	4+							
	1-4000							1+			4+
	1-10000								1+		2+
	1-20000					no growth					
Filtrates of yolk infusions (Coors)	1-10	1+	4+								
	1-30		1+			4+					
	1-100					no growth					

The inoculum in each case was 10-8 mg of moist growth (about 10 tubercle bacilli). The data given are the averages of from 6 to 20 experiments.

The N-Z amine gave better growth with some of the yolk mediums than did asparagine, hence was preferred. The pH for optimal growth is not critical; reactions between pH 6.0 and 7.5 seem equally satisfactory. The yolk has a pH of about 6.0 and is not heavily buffered, so that adjusting the diluting fluid to the desired pH is usually all that is necessary.

Preparation of the inocula. Four different strains of human type tubercle bacilli were used; 3 of these were freshly isolated; the fourth has been cultivated for 6 years. Every significant experiment was repeated with all 4 strains; all 4 behaved essentially the same.

Ten to 20 day old cultures on solid medium were ground up in a tissue grinder with a few ml of the Dubos Tween-albumen medium. Clumps were removed by centrifugation at 1000 r.p.m. for 6 minutes; the supernatant suspension was diluted to a density equal to that of tube No. 3 of McFarland's standards (6). Such a suspension contains 10^0 mg of moist tubercle bacilli per ml, or 1 billion single

viable organisms. Microscopic examination showed that the bacilli were mostly single with a few small clumps.

Small tubes, each containing 1.5 ml of the medium, were inoculated in duplicate with doses of 10^{-4} , 10^{-6} , and 10^{-8} mg of moist culture contained in a volume of 0.1 ml. Colony counts were always made; a dose of 10^{-8} mg usually grew from 7 to 20 colonies. Cultures were incubated at 37 to 38°C. Growth in the cultures was determined by making smears at appropriate intervals. As growth is entirely subsurface, the smears were taken from the sediment. Scanty growth was recorded as 1+; numerous cords or clumps in every low power field as 4+. Incubation was continued for 35 days.

Egg yolk mediums. 1. Egg yolk diluted with distilled water and autoclaved. When egg yolk is diluted and autoclaved at the usual pH, a coagulum is formed. If the dilution is 10-fold or greater, the most of the medium is liquid. Table I shows that when yolk is diluted 1 to 10 with distilled water and autoclaved, this medium supports growth of the tubercle bacillus very well, even when the inoculum is as small as 10^{-8} mg (about 10

5. Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., *Am. Rev. Tuberc.*, 1946, v54, 204.

6. McFarland, J., *J. A. M. A.*, 1907, v49, 1176.

organisms). Growth is fully as rapid and profuse as with the Dubos liquid mediums. Dilutions of yolk up to 1 to 100 still support good growth, but higher dilutions are unsatisfactory. With larger inocula, such as 10^{-4} mg, smears from yolk dilutions up to 1-100 become positive in 5 days and heavily positive within 9 days; with this inoculum, yolk dilutions up to 1-600 will give growth.

2. *Egg yolk diluted with basal medium and autoclaved.* When yolk is diluted with the basal medium instead of with water, far less yolk is needed to obtain growth of the tubercle bacillus. As Table I indicates, as little as 1 part of autoclaved yolk in 2500 parts of basal medium has a demonstrable growth stimulating effect with inocula of 10^{-8} mg, while 1 part in 5000 is effective with inocula of 10^{-6} mg or more. When the inoculum is 10^{-4} mg, smears are positive in 4 to 6 days in yolk dilutions up to 1-250. The basal medium alone, without yolk, supports growth only when heavily seeded; with an inoculum of 10^{-4} mg sub-surface growth occurs in about 20 to 24 days and becomes moderately heavy in 35 days. Smaller inocula never grew out in the basal medium. In these autoclaved mediums, the growth stimulating substances are associated with the coagulum. If the fluid portion is completely freed from particulate matter (as by filtration through a Coors filter, or even through a retentive filter paper), it will not support growth. After centrifugation at 5000 r.p.m., supernatant fluids may sometimes support growth because all particulate matter is not removed. If the supernatant fluid is removed and replaced by adding sterile water or basal medium to the coagulated material, growth will occur. If a small amount of yolk (best diluted with an equal volume of water) is coagulated as a slant and covered with sterile water or basal medium, growth occurs in the liquid phase. In the autoclaved mediums, the organisms occur chiefly in loose clumps which often become completely dispersed and appear as clouds of single bacilli. Bacilli in "cords" as described by Middlebrook, Dubos, and Pierce (7) may also appear.

3. *Unheated sterile egg yolk diluted with basal medium.* Unheated egg yolk is least soluble in distilled water and most soluble in 10% sodium chloride solution in the pH range used for these mediums. When diluted with the basal medium, much of it remains insoluble and settles out. The further addition of diluent does not bring more yolk into solution; instead, solid matter is precipitated out of the liquid phase. This indicates the presence of some powerful dispersing agent, the effectiveness of which is diminished by dilution. When the insoluble material is removed by centrifugation, the supernatant fluid contains active substances, though growth is usually better when the insoluble material is left in. In the experiments shown in Table I, the sediments were not removed. With mediums prepared in this way, the activity of unheated yolk can be demonstrated in dilutions as high as 1 part in 20,000 with inocula of 10^{-6} or greater, or 1 part in 10,000 with inocula of 10^{-8} mg. When the yolk content is 1 or more parts per 1000, growth is rapid and profuse, even with the smallest inocula (Table I). When raw egg yolk is diluted with distilled water, growth is both slower and less luxuriant than when the same medium is autoclaved. Yolk diluted with the salts only of the basal medium (without the asparagine or N-Z Amine) supports growth well. The grouping of the tubercle bacilli in smears from these unheated yolk mediums is quite different from the loose clumps found with the autoclaved yolk. Here the arrangement is like that seen with the Dubos Tween-albumen medium: cords of bacilli, often very long and thick; also matted filaments or "Medusa heads" (7).

4. *Filtrates of unheated yolk infusions.* When egg yolk is diluted and infused with the basal medium (over night in the refrigerator, then 3 hours at 45°C), then centrifugated and filtered through a Coors filter, a strongly opalescent filtrate is obtained. If the filtrate from a 1 in 10 or 1 in 20 infusion is diluted further, a precipitate forms; this precipitate is mostly lipid but contains protein also. It is least soluble in distilled water and is more soluble in salt solutions. The precipitate contains most of the growth stimulating factors.

7. Middlebrook, G., Dubos, R. J., and Pierce, C., *J. Exp. Med.*, 1947, v86, 175.

The filtrates from the more concentrated infusions in basal medium are excellent growth mediums for the tubercle bacillus (Table I). If more dilute infusions (e.g., 1 part or less of yolk in 100 parts of basal medium) are made, very little of the growth stimulating material goes into solution and the filtrates do not support growth well.

These filtrates, while free from gross particles, are opalescent emulsions. An effort was made to secure a yolk filtrate that was water clear and would yet support vigorous growth of small inocula. This was done by extracting egg yolk repeatedly with acetone at room temperature; drying and powdering the residue; then making an infusion of the powder (0.66 g in 25 ml of basal medium) and filtering through a Coors filter. The filtrate was water clear. With an inoculum of 10^{-4} mg, smears were positive in 5 days and heavily positive in 8 days. With an inoculum of 10^{-8} mg smears were positive in 18 days and growth was heavy in 25 days. When the acetone-extracted yolk was further extracted with hot alcohol (which removes the phospholipids), the aqueous extract of the residue did not support the growth of tubercle bacilli. The fraction removed by the hot alcohol, when added to basal medium and autoclaved, did support growth. That the phospholipid fraction of egg yolk is largely

responsible for the extraordinary capacity of the yolk to stimulate the growth of tubercle bacilli is clear from the reported observations of Boissevain and Schultz(8), Dubos(9), Finlayson(10), and others. Sphingomyelin, which constitutes about 0.3% of the weight of the yolk, is known to enhance the growth of the tubercle bacillus(11). Experiments still in progress indicate that other constituents besides the phospholipids may be equally important.

Summary. 1. Egg yolk can be used as the basis for several easily prepared liquid and semi-liquid mediums.

2. On such mediums, growth of the tubercle bacillus is rapid and heavy, even from small inocula.

3. By first removing the acetone soluble fats, water clear extracts of egg yolk can be prepared; these will support growth from small inocula.

8. Boissevain, C. H., and Schultz, H. W., *Am. Rev. Tuberc.*, 1938, v38, 624.

9. Dubos, R. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, v58, 361.

10. Finlayson, M. K., *J. Path. Bact.*, 1946, v58, 88.

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A Radioautographic Method for Detailed Localization of Radioactive Isotopes in Tissues without Isotope Loss. (17739)

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Animal tissues are prepared for radioautographs by freezing in isopentane at -170°C followed by vacuum dehydration, infiltration with paraffin under vacuum, cutting with the technic described previously(1), and direct mounting of the section on a microscope slide or a thin cover glass. The tissue

holder (slide or cover glass) is then placed in a vertical position in an oven at approximately 45°C ; the paraffin liquefies and all excess paraffin is drained from the slide and tissue section. An alternative method is to remove the paraffin more completely by means of a paraffin solvent such as xylol, warm dioxane, or warm absolute alcohol. The tissue is returned to the oven for a suitable length of time to ensure complete evaporation of the

1. Holt, M. W., Cowing, R. F., and Warren, S., *Science*, 1949, v110, 328.

paraffin solvent (if one is used) before the section is attached to the photographic plate. The results obtained to date have indicated that use of the above-mentioned paraffin solvents does not produce appreciable variations in radio-autographs obtained from frozen-dehydrated tissues with I^{131} . This is being investigated further. As was reported previously (1) for frozen-dehydrated tissues with P^{32} , placing the tissue containing I^{131} in water does result in loss of a major portion of the activity. These results have been obtained for varying time intervals (such as 2, 4, 24, and 48 hours) from injection of the isotope to killing the animal.

The photographic exposure is made by clamping the tissue slide or cover glass face to face with the emulsion side of a photographic plate. In order to prevent direct contact of the tissue with the emulsion surface a thin plastic "spacer" is used. This is a ring of polystyrene 1 mil in thickness which encircles the tissue section; the diameter of the opening is approximately 1 cm. The rings are cut from sheet polystyrene by means of a special punch devised for this purpose. Since the tissue is ordinarily cut at a thickness of $8\ \mu$, the use of the plastic spacer provides an air gap of approximately $18\ \mu$ between the tissue surface and the emulsion. The spacer is considered to be desirable because it eliminates the possibility of direct chemical action of the tissue on the emulsion, and it permits easy separation of the two slides at the end of the exposure. The tissue slide or cover glass and photographic plate are clamped together (using small binder clips or other suitable clamps) and stored for exposure. As will be pointed out later, mounting the tissue sections on a cover glass is preferable if localizations are to be studied at high magnifications. After a suitable exposure time, the two slides are separated; the photographic plate is developed and the tissue slide is stained. The tissue slide and radioautograph are both examined under the microscope and photomicrographs are made of the corresponding images in areas of interest. The eyepiece of the microscope is fitted with a ruled micrometer disc which is adjusted in position to give a sharp focus at the photographic plate.

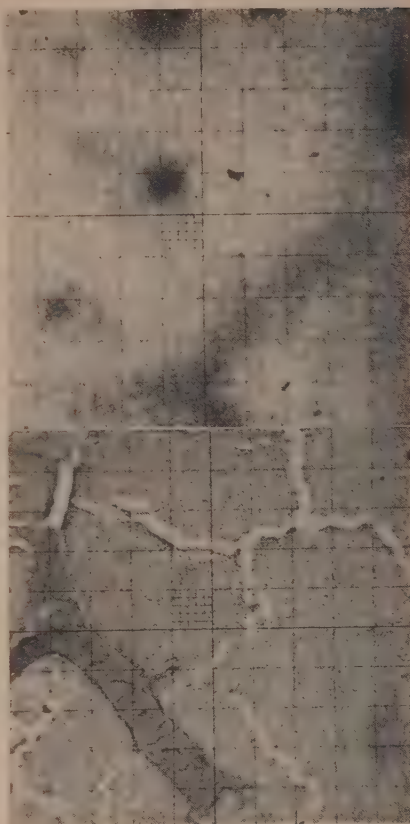


FIG. 1.

a (bottom), Hematoxylin stained $8\ \mu$ section of frozen dehydrated liver from a 135 g rat injected with $48\ \mu\text{c}$ carrier free I^{131} 3 hours before sacrifice; *b* (top), corresponding radioautograph on Eastman Kodak NTB-3 $50\ \mu$ plate, 17-day exposure started 12 days after the injection. Magnification $50\times$.

The photographs illustrate the method used in locating regions of the tissue that correspond to numerous as yet unexplained "hot spots" observed in the liver of this animal.

The coordinate ruling, which photographs with the tissue image, is very useful in matching corresponding regions of the two resulting photographs (Fig. 1). In locating corresponding positions on tissue and radioautograph slides, it is necessary to have a coincident reference mark on the two slides which can be either within the field to be photographed or outside. The use of a reference mark within the field is illustrated by the large blood vessel shown in Fig. 1. The procedure used was



FIG. 2.

a (left), Hematoxylin stained section ($8\ \mu$) of frozen dehydrated liver from a 122 g rat injected with $500\ \mu\text{c}$ carrier P^{32} two hours before sacrifice. *b* (right), Corresponding radioautograph on Eastman Kodak NTB-3 $50\ \mu$ plate, 21-hour exposure started 8 days after the injection. X marks made with ink containing $4.4\ \mu\text{c}\ \text{P}^{32}/\text{cc}$. Magnification $3\times$.

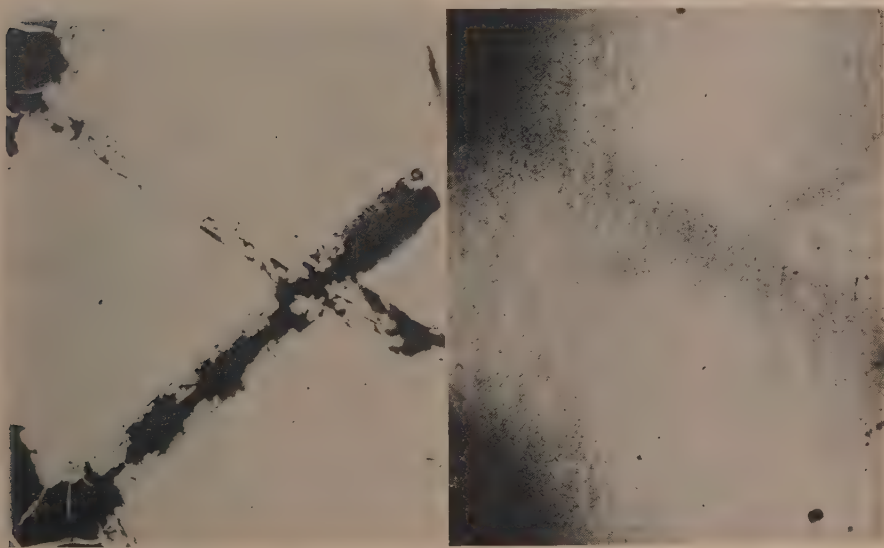


FIG. 3.

a (left), A very small ink X mark which was made by placing two dots of ink on the slide and pulling an extremely fine glass capillary through the ink dot and along the slide before the ink in the dot had been allowed to dry. With this method, lines approximately $50\ \mu$ wide can be made. *b* (right), Radioautograph obtained from the ink mark illustrated in *a* shown as a mirror image. The ink used contained approximately $200\ \mu\text{c}\ \text{P}^{32}/\text{cc}$ and the exposure time with NTB-3 plates was 3 hours. Magnification $50\times$.

to adjust the position of the reference mark on each slide to coincide with a definite position on the eyepiece ruling before photographing.

With high magnifications, it is necessary to

use reference marks located outside the microscope field. This can be accomplished by employing a calibrated mechanical stage on the microscope in combination with either the ocular micrometer ruling or a cross-hair in the

observation eyepiece of the photomicrographic camera. The reference mark is centered with the cross-hair or micrometer ruling and the position of the slide noted; the slide is then moved to the object to be photographed and the procedure repeated. The reference mark on the second slide is then located, the slide is moved to the position corresponding to that of the object photographed on the first slide (as determined by the calibrated stage) and this region is photographed.

In order to mark slides, a radioactive ink has been developed. The ink is prepared by adding an appropriate amount of carrier free P^{32} to glass marking ink (Clay-Adams Gold Seal black ink was used). Photographs showing the type of marks used and the corresponding radio-autographs are shown in Fig. 2 and 3; a description of the method used in preparing the marks is included under the figure caption. The precision of this method has been tested by using one of the ink marks as a reference mark and locating the position of other marks on the slide and radioautograph. It was found that the accuracy in determining the position of the marks on the radioautographs with respect to one another and to the tissue slide marks was as good as the accuracy of the mechanical stages tested (to within $100\ \mu$ with an ordinary mechanical stage and to within $25\ \mu$ using a modified mechanical stage with this degree of precision).

Since the radioautograph obtained with this technic is a mirror image of the tissue section, it is usually desirable to photograph the tissue section upside down(2). This makes the images correspond and simplifies comparisons of the photomicrographs. In order to focus on the inverted tissue section at high magnifications, it is necessary for the section to be mounted on a thin cover glass. After the photographic exposure has been made and the sections have been stained, the cover glass with attached sections is mounted with balsam on an ordinary slide. In this way, mirror images are avoided, and when the slides are observed in the usual manner the orientation of the tissue sections corresponds directly to the radioautographs.

In making further refinements of the method, the following factors will determine the accuracy to be attained: (1) limitations due to the size of the reference marks used; (2) the accuracy of the mechanical stage (further refinement can readily increase this precision to $\pm 2\ \mu$); (3) the distance separating the tissue and the emulsion surface; and (4) the resolution of the emulsion (an emulsion thickness of around $10\ \mu$ or less should improve this factor). It would also be desirable to decrease the thickness of the tissue section and to minimize back-scattering from the slide surfaces.

The basic principle of the method of making radioautographic exposures described in this paper (*i.e.* facing the specimen to the photographic plate followed by separation of the specimen and the plate at the end of the exposure) was originated by the work of London(3) and of Lacassagne and Lattes(4) and has been used in many studies. The primary objections to the method have been due to lack of sharpness of the radioautographs and to difficulties in matching the radioautograph with histological details(5). A method of matching the tissue slide and radioautograph which is somewhat similar to the one described here has been reported previously by Boyd (2). This technic employs a double microscope comparator with a mechanical stage common to two microscopes. A reference mark is prepared by taking a shadowgraph of an 8-10 μ piece of gold leaf which is placed near the tissue section.

The procedure of preparing radioautographs by painting the tissue section with liquid photographic emulsion, as described by Belanger and Leblond(6) was tried in this laboratory. Some difficulties were experienced with this method (such as those mentioned by Evans(5)); also, in working with frozen-dehydrated tissues with isotope concentrations

3. London, E. S., *Arch. d'electric. med.*, 1904, v12, 363.

4. Lacassagne, M. A., and Lattes, J. S., *C. R. Acad. d. sc.*, 1924, v178, 488.

5. Evans, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, v64, 313.

6. Belanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, v39, 8.

2. Boyd, G. A., *MDDC*, 1226.

comparable to those reported previously(1), a blurred radioautograph resulted. This was due to loss of the isotope into the liquid emulsion. The method described by Endicott(7) and Evans(5) of permanently mounting the tissue section directly on the photographic emulsion offers the advantage of intimate contact of the tissue section and the emulsion with corresponding gain in resolution. However, the question of possible chemical fogging of the emulsion by the tissue does arise. This method is also subject to staining difficulties. Conventional mounting technics are employed (floating the tissue on water) and it would not be easily adaptable to the cutting and mounting technic developed in this laboratory for avoiding isotope loss because of the problem of removing the paraffin.

The use of stripping film(8,9) for preparing radioautographs avoids staining difficulties when the tissue is mounted on the photographically insensitive side. However, it is more complicated to handle than an ordinary slide; the tissue must be mounted in the darkroom; and the tissue is separated from the emulsion by a 10 μ cellulose ester (which probably results in more absorption and scattering of radiation than would be produced by 18 μ of air). It does have the advantage of minimizing back-scattering.

Although there is partial separation of the tissue image and the radioautograph because of difference in the focal plane in the stripping film method, these latter 3 methods all

have the disadvantage of super-position of images. This makes it difficult to see details of either one individually, especially where the tissue section is stained.

Summary. The method described in this paper incorporates the following desirable features:

1. It is possible to prepare radioautographs from tissue that has been frozen and dehydrated in vacuum and mounted by procedures that avoid contact of the tissue with reagents that would extract the radioactive material.
2. Sharp images are obtained; yet the method avoids possible difficulties due to direct chemical contact of the tissue with the photographic emulsion.
3. There are no difficulties in staining and developing, since the two slides are handled separately.
4. Due to the fact that two images are on separate slides, the photographic image formed does not obscure structural details of the tissue section or vice versa; comparisons are always made on the same tissue section that was used in making the photographic exposure.
5. It is possible to match localizations accurately on the two slides.

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Effect of Niacin Precursors on the Excretion of Niacin Metabolites.* (17740)

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The interchangeability of tryptophan and niacin in the nutrition of rats receiving rations low in protein has indicated the utilization of this amino acid in the synthesis of the vitamin(1-3). It has been shown in work with *Neurospora*(4,5) and later with animals(6,7) that kynurenine, hydroxy-kynurenine and hydroxy-anthranilic acid are intermediates in this conversion. Investigations by Ellinger *et al.*(8) have shown that an increase in the nicotinamide content of suspensions of rat cecal contents occurs when glutamic acid, arginine or ornithine was added to the media. On the basis of these observations, they suggested that nicotinamide was synthesized from glutamic acid and arginine with ornithine as an intermediate. The observation by Teply *et al.*(9), that the type of carbohydrate in the diet markedly influenced the niacin level of

rat cecal contents, indicated some synthesis of niacin by the intestinal bacteria. However, work with enterectomized rats has suggested that a larger percentage of the synthesis occurs in the tissues(10). Further work with isolated tissues has indicated the liver and kidneys of the rat are the possible sites of synthesis(11).

Recently Henderson(12) found that quinolinic acid was the compound in rat urine, which on acid treatment (decarboxylation) gave niacin activity for *L. arabinosus*. On the basis of this observation he suggested that quinolinic acid was probably the intermediate compound between hydroxy-anthranilic acid and niacin. Further work by Henderson(13) has shown that the incubation of liver homogenates with added 3-hydroxy-anthranilic acid caused an increase in the quinolinic acid content of the suspension. Additional experiments by Bonner(14) with mutant strains of *Neurospora* have shown that one of the mutants, grown in the presence of 3-hydroxy-anthranilic acid, can also accumulate quinolinic acid. However, these workers have not been able to find a mutant of *Neurospora* which can utilize quinolinic acid. Bonner suggests that quinolinic acid is not the intermediate compound, but that after the 3-hydroxy-anthranilic acid ring is split in the 3,4 position a decarboxylation occurs with subsequent ring closure to form niacin.

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14. Bonner, D. M., and Yanofsky, Charles, *Proc. Nat. Acad. Sci.*, 1949, v35, 576.

In addition to the compounds proposed as intermediates in synthesis of niacin another possible intermediate between 3-hydroxy-anthranilic acid and niacin is isocinchomeronic acid, which would be the dicarboxylic acid obtained if hydroxy-anthranilic acid had been split in the 2,3 position. A study was therefore conducted to determine the actual extent of synthesis of niacin and its metabolites in the intact animals, when these compounds were fed.

Experimental. Rats of the Sprague-Dawley strain weighing 170-290 g were placed in individual metabolism cages for a period of several weeks and fed (*ad libitum*) a 9% casein basal which has been used repeatedly for tryptophan and niacin studies(11). Control samples of urine and feces were collected from each animal over a 3-day period and then the animals were divided into groups of three. After the feeding of the compounds under study, three-day samples of urine and feces were again collected from each animal. Three-day periods were used because previous work in this laboratory(10) showed that after an injection of a massive dose (300 mg) of tryptophan the urine niacin values returned to normal within this time. The compounds fed were anthranilic acid, ornithine, quinolinic acid, isocinchomeronic acid, tryptophan and anthranilic acid plus ornithine.

The isocinchomeronic acid was made by the oxidation of quinaldine according to the method of Soine(15), while the other compounds were obtained commercially. All compounds were fed on an equimolar basis with 50 mg of tryptophan. In the first experiment the compounds (in aqueous solution) were given by stomach tube to the animals in 2½ ml doses, which were spaced three hours apart. In a second experiment the compounds were given in a single, 5 ml dose. The urine samples were collected under toluene. At the end of the collection periods, they were made up to volume, filtered and stored at 5°C under toluene until analyzed.

N-methyl nicotinamide was determined by the acetone-fluorometric method of Huff *et*

al.(16). Another portion of each urine was neutralized, diluted, and assayed for niacin by the microbiological assay(17). This value was termed "free" niacin. Work by Henderson(18) has shown that the complete decarboxylation of quinolinic acid in urine is obtained by treatment with glacial acetic acid. Therefore, a third portion of each urine (2 ml) was evaporated to dryness and autoclaved for 3.5 hours at 15 pounds pressure with 0.5 ml of glacial acetic acid. After neutralizing, "total" niacin was determined microbiologically(17). Samples of feces (0.2 g) from the control animals were hydrolyzed by autoclaving for 3.5 hours at 15 lb pressure with 0.5 ml of glacial acetic acid. All feces from the experimental period were ground in a mortar before sampling and then given the same treatment for determination of "total" niacin content. The compounds used in this study gave no activity for niacin when assayed with *L. arabinosus*.

Results and discussion. An increase in the free niacin content of the urine excreted by all animals could be detected after the feeding of the various metabolites (Table I). However, this increase was not significant in the case of ornithine, anthranilic acid or isocinchomeronic acid. It is evident that the increase in free niacin excretion due to the feeding of quinolinic acid was not as great as that obtained with an equimolar quantity of tryptophan. A much greater increase in free niacin should be obtained with this metabolite, if it were a direct precursor of niacin. On the other hand it is possible that the quantity of quinolinic acid administered was too large and it had a depressing effect on the enzyme system converting quinolinic acid to niacin. The much higher excretion of free niacin obtained after tryptophan administration was probably due to the fact that the chain of reactions for niacin synthesis from this amino acid is long enough to prevent any toxic ef-

16. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, v167, 157.

17. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, 1943, v15, 471.

18. Henderson, L. M., and Hirsch, H. M., *J. Biol. Chem.*, 1949, v181, 667.

15. Soine, T. O., *Am. Pharm. Assn.*, 1944, v33, 223.

TABLE I.
Effect of Precursors on Excretion of Nicotinic Acid and Its Derivatives by Rats.*

Supplement	Animal wt	$\mu\text{g per 24 hr}$					
		Urinary excretion			Fecal excretion		
		Free nicotinic acid		Total nicotinic acid after acid treatment	N'methyl- nicotinamide†		Total nicotinic acid after acid treatment
		Control	Metabolite		Control	Metabolite	
30 mg tryptophan	239	14	56	46	1427	272	14.8
41 mg quinolinic acid	210	12.8	26.8	41	1344	456	19.4
32.4 mg ornithine	252	12.6	15.5	62	64	47	1539
41 mg isocinchomeric acid	272	13	20	57	208	164	29
33.5 mg anthranilic acid	217	13.2	15	59	68	207	17
33.5 mg anthranilic acid + 32.4 mg ornithine	218	10.4	15.8	64	62	153	20
							18.4

* All values in table are the average of 3 animals.

† Grateful acknowledgment is made to Richard L. Lyman for conducting the N'methyl nicotinamide determinations.

fects of a massive dose of the starting material. These results compare very well with excretion data obtained by Henderson (16). In his experiments he compared the effect of daily injection of equimolar quantities of tryptophan, 3-hydroxy-anthranilic acid and quinolinic acid.

It is evident that the material in urine active for *L. arabinosus*, after acid treatment (quinolinic acid), was markedly increased when either tryptophan, quinolinic acid or isocinchomeric acid was administered. The rather large increase after the feeding of tryptophan or quinolinic acid could be due to a limiting rate of reaction in the formation of niacin from quinolinic acid. The decarboxylation of quinolinic acid to niacin could be a much slower reaction than any of those preceding it, therefore the excess quinolinic acid spills over into the urine. This theory apparently can be applied only to tryptophan and quinolinic acid. Preliminary work with isocinchomeric acid indicated that the autoclaving with glacial acetic acid did not alter (decarboxylate) the isocinchomeric acid molecule. This compound gave no niacin activity for *L. arabinosus* either before or after treatment with glacial acetic acid. With this thought in mind it is evident that the increase in the total niacin value after the feeding of isocinchomeric acid, could not be the result of decarboxylation of isocinchomeric acid, but must have come from the decarboxylation of quinolinic acid. Apparently the isocinchomeric acid either stimulated the enzyme system involved in the formation of quinolinic acid or it protected the quinolinic acid from destruction by the tissue enzymes.

The N'methyl nicotinamide content of the urines increased 15 to 40 times over the controls after the administration of the various metabolites. Again the increases obtained with tryptophan, quinolinic acid and isocinchomeric acid were the greatest. However, in this case, quinolinic acid gave a much higher value than tryptophan. It is possible that the animal metabolized some of the quinolinic acid to nicotinamide and then to N'methyl nicotinamide. On the other hand, it is also possible that the massive dose of quinolinic acid administered to the animals caused

the formation of a methylated quinolinic acid, which was active in the fluorometric method used. It is interesting that several of the compounds caused increases in the excretion of N'methyl nicotinamide without a corresponding increase in the free and total niacin. Apparently these compounds stimulate the formation of a compound in urine which is active in the N'methyl nicotinamide determination.

The analyses of the feces for nicotinic acid revealed that the influence of the various compounds in the intestinal tract was quite variable. Increases in fecal niacin occurred with the feeding of all compounds except isocinchomeric acid. Evidently the high level of this compound in the diet was toxic to the bacteria in the intestinal tract, and it depressed the synthesis of the vitamin. If the increases in the fecal niacin obtained in the anthranilic acid and ornithine groups are considered significant, these compounds undoubtedly acted by stimulation of the bacteria in the intestinal tract. It may also be suggested that these two compounds are apparently utilized by the bacteria in the tract and not by the tissues for the synthesis of the vitamin. The niacin synthesized in the tract in this case could be in a bound (unavailable) form. The high fecal niacin value for the animals receiving quinolinic acid was obviously due to the quantity of the compound that was not absorbed. However, this quantity amounts to only about one-eighth of the quinolinic acid fed and indicated that the compound is fairly well absorbed. If the increase in the various niacin metabolites in the urine and in the feces of the quinolinic acid group are summated and calculated back to quinolinic acid, the value represents only 30% of the quinolinic acid fed, which indicates that about 70% of it was metabolized by the animal. Whether it was metabolized as niacin or passed out into the urine as some other metabolite such as the pyridone(19) is not known.

The data obtained in the second experiment in which the compounds were fed in a single 5 ml dose compared very well with the data already discussed. The only variation was a slightly lowered excretion of N'methyl nicotinamide for those animals receiving quinolinic acid.

It is evident from the data reported that isocinchomeric acid is not involved in the synthesis of niacin. Ornithine and anthranilic acid apparently have some stimulatory effect on the bacteria in the tract which synthesize the vitamin. Considering the effect of these two compounds on the free niacin in the urine, evidently they do not influence the synthesis of the vitamin in the tissues. The data presented on quinolinic acid neither prove nor disprove that this compound is a possible intermediate between 3-hydroxy-anthranilic acid and niacin. However, it is evident that quinolinic acid is utilized by some enzyme system, which may be hindered by the presence of isocinchomeric acid, and thereby cause the accumulation of quinolinic acid, which is eventually excreted. The combination of anthranilic acid plus ornithine was fed, because growth data in other experiments showed that the combination of these two compounds overcame the growth reduction due to the supplementation of 0.2% DL-phenylalanine to a 9% casein ration(20).

Conclusions. 1. The effect of tryptophan, quinolinic acid, ornithine, isocinchomeric acid, and anthranilic acid on the ability of the rat to form niacin derivatives has been determined.

2. Isocinchomeric acid does not appear to be an intermediate in the synthesis of niacin.

3. Ornithine and anthranilic acid seem to stimulate the synthesis of niacin in the intestinal tract only.

4. The results neither prove nor refute the theory that quinolinic acid is a possible intermediate in the tryptophan niacin synthesis scheme.

20. Hanks, L. V., unpublished data.

19. Knox, E. W., and Grossman, W. I., *J. Biol. Chem.*, 1946, v166, 391.

Effect of Some Surface Active Agents on the Action of Barbiturates. (17741)

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Interest in the effect of surface active agents on drugs stems mainly from their inhibitory action on enzyme systems(1-5), although penetration can not be entirely disregarded as a factor. Eiseman(6) by *in vitro* experiments has shown that by combining a known tuberculostatic agent with a surface active agent the tuberculostatic action was increased. The study described below was undertaken to investigate the effects of these agents on barbiturates. The doses of surface active agents used were such that no observable effect was produced. Pentobarbital and thiopental were

used as examples of those barbiturates which are destroyed in the liver(7,8). Barbitol was used as an example of a barbiturate not destroyed in the body(7). The onset of hypnosis was considered to be the loss of righting reflex and the duration the time between loss of righting reflex and the regaining of the reflex.

Experiments with Pentobarbital Sodium.

Eleven surface active agents were selected from the group which had been tested for toxicity(9), and one-half the LD₅₀ was given with a hypnotic dose of sodium pentobarbital

TABLE I.

Copyright and Chemical Names of the Agents Studied with One-half the Intravenous LD-50 for Mice. The amounts stated represent the amounts of active material contained in the commercial preparations.

Agents	Type	½ LD-50, mg/kg	Chemical names
Emulsept	C	10	Lauric and myristic acid esters of colaminoformylmethyl-pyridinium chloride
Igepal CA	N	35	Polymerized ethylene oxide condensate
Aerosol O.S.	A	60	Isopropyl-naphthalene sodium sulfonate
Santomerse 3	A	52	Dodecyl benzene sodium sulfonate
Areskap 100	A	90	Monobutylphenyl-phenol sodium monosulfonate
Aresket 300	A	125	Monobutyl diphenyl sodium monosulfonate
CDMEN Br.*	C	25	Cetyl dimethyl ethyl ammonium bromide
Aerosol O.T.	A	30	Dioctyl ester of sodium sulfosuccinic acid
Emcol 888	C	4	Polyalkyl naphthalene pyridinium chloride, average M.W. 382
Santomerse D	A	60	Decyl benzene sodium sulfonate
Aresklene 400	A	100	Dibutylphenyl-phenol sodium disulfonate

* Our abbreviation.

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2. Kirsner, J. B., and Wolff, R. A., *Gastroenterology*, 1944, v2, 270.
3. Kirsner, J. B., and Spitzer, E. H., *Gastroenterology*, 1944, v2, 348.
4. Block, C. L., Portis, S. A., and Necheles, H., *Gastroenterology*, 1944, v3, 45.
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6. Eiseman, B., *J. Exp. Med.*, 1948, v88, 189.
7. Dorfman, A., and Goldbaum, L. R., *J. Pharm. and Exp. Ther.*, 1947, v90, 330.
8. Shideman, F. E., Kelly, A. R., and Adams, B. J., *J. Pharm. and Exp. Ther.*, 1947, v91, 331.
9. Hopper, S. H., Hulpieu, H. R., and Cole, V. V., *J. Am. Pharm. Assn.*, 1949, v38, 428.

TABLE II.

The Effect of Surface Active Agents ($\frac{1}{2}$ LD₅₀) on the Sleeping Time of Mice Given an Intravenous Dose of 50 mg/kg of Sodium Pentobarbital. The surface active agents were mixed with the sodium pentobarbital.

Surface active agent	No. lived	Duration of hypnosis (min.)	
		Mean	S.E. _m
None	20	23.3	2.08
Emulsept*	20	32.2	2.16
Igepal CA*	19	33.1	3.78
Aerosol O.S.	20	34.0	1.17
Santomerse 3	20	34.4	2.65
Areskap 100	20	35.8	4.27
Aresket 300	19	37.0	3.35
CDMEN Br.*	20	38.4	3.22
Aerosol O.T.*	20	39.4	3.82
Emcol 888*	16	41.0	4.53
Santomerse D*	17	46.1	5.23
Aresklene 400*	18	47.7	3.37

* These agents were retested giving the surface active agents 5 min. before the pentobarbital.

to groups of mice. From this group of 11, one was selected which gave a typical effect and that one was studied on rats, rabbits and dogs. The mice used were all female; the rats and rabbits used were male; the dogs were both male and female with matched pairs between the control and those receiving the surface active agent. Table I gives a list of the agents studied with the ionic type, the chemical nature and one-half the LD₅₀. Table II shows the experiments on mice which received 50 mg per kg of sodium pentobarbital alone and mixed with surface active agents. Twenty mice were used in each experiment. In all instances where a surface active agent was given the duration of hypnosis was longer with a surface active agent than without. In order to rule out the possibility of a reaction between the surface active agents and sodium pentobarbital, mice were given a surface active agent and 5 minutes later the same dose of sodium pentobarbital was given. Seven compounds were so tested. Qualitatively, the same results were obtained as before and 6 of the agents gave the same quantitative results. Santomerse D showed a significantly longer time of hypnosis when the surface active agent was given before sodium pentobarbital than when it was given at the same time. Thus, the effects observed in our first experiment were not due to the barbiturate

being mixed with the surface active agent. Twenty male rats were then given 30 mg per kg of sodium pentobarbital by intraperitoneal injection. At the time of the loss of righting reflex Aresklene 400 was given intravenously to 10 of these in the dose of 50 mg per kg. The mean time of hypnosis was increased from 66.1 to 118.1 by Aresklene 400. Sodium pentobarbital 30 mg per kg was administered intravenously to male rabbits with and without Aresklene 400, 50 mg per kg. All the controls survived but 5 out of 10 rabbits which received the Aresklene died. There was no difference in sleeping time between the survivors and the controls. Four pairs of dogs matched for sex and weight received 25 mg per kg of sodium pentobarbital. One of each pair received Aresklene 400, 25 mg per kg or one-eighth the LD₅₀. Differences between numbers of pairs were small and inconsistent.

Experiments with Thiopental Sodium.

Female mice were used for these experiments and thiopental sodium was given intravenously in a dose of 50 mg per kg. The surface active agents were tried in doses of one-half the LD₅₀, but it was found that this dose caused an increase in death rate. Therefore, the experiment was conducted with one-fourth the LD₅₀ of surface active agents. The same 11 compounds were studied as were studied with sodium pentobarbital. Table III shows the

TABLE III.

The Effect of Surface Active Agents ($\frac{1}{4}$ LD₅₀) on the Death Rate and Sleeping Time of Mice Given an Intravenous Dose of 50 mg/kg of Sodium Thiopental. The surface active agents were mixed with sodium thiopental.

	No. of mice	% died	Duration of hypnosis (min.)	
			Mean	S.E. _m
Control	15	6.7	8.0	0.56
Santomerse 3	10	50	5.6	2.12
CDMEN Br.	10	0	7.6	0.68
Aerosol O.S.	10	60	9.5	3.53
Santomerse D	10	0	10.0	1.59
Emulsept	10	30	10.4	2.66
Emcol 888	10	40	10.5	1.34
Igepal CA	10	10	13.0	1.68
Areskap 100	10	20	16.7	2.84
Aresklene 400	20	20	19.6	2.92
Aresket 300	10	20	20.1	5.60
Aerosol O.T.	20	10	23.8	3.23

TABLE IV.

The Recovery of Thiopental Sodium Administered Intravenously to Mice With and Without Aresklene 400 ($\frac{1}{4}$ LD-50).

	Controls	Aresklene 400, 50 mg/kg
	30 min. after inj.	
No. of mice	13	14
% thiopental recovered		
Mean	66.3	77.7
S.E. _m	1.69	2.89
	120 min. after inj.	
No. of mice	10	10
% thiopental recovered		
Mean	57.7	69.3
S.E. _m	2.70	3.18

10). The range given in per cent recovered to that injected varied at 30 minutes with the controls from 53.1 to 75.0, while in those receiving the surface active agent the variation was from 61.0 to 102.0. The chance is less than 1 in 100 that the difference was not significant. At two hours the range for controls was 44 to 72%, for those receiving Aresklene the range was 54 to 85%. The chance is less than 1 in 50 that this difference was not significant. It seems probable that the interference with the destruction of the thiopental accounts for the longer sleeping time.

Experiments with Barbitol Sodium. Twen-

TABLE V.

The Effect of Surface Active Agents on the Onset of Hypnosis of Mice Receiving Intravenously 300 mg/kg of Barbitol Sodium. The surface active agents were given mixed with the barbitol sodium.

	No. of mice	No. of mice anesthetized	Onset of hypnosis (min.)	
			Mean	S.E. _m
Control	20	19	33.9	3.23
Emulsept	10	10	37.2	3.21
Aerosol O.T.	10	10	34.8	4.10
Santomerse D	10	10	30.1	3.97
Aresket 300	10	8	30.1	6.30
Areskap 100	10	10	21.4	2.47
Santomerse 3	10	10	19.7	2.76
Emcol 888	10	7	16.3	1.19
Aerosol O.S.	10	10	12.2	1.27
CDMEN Br.	10	10	3.7	1.24
Igepal CA	10	10	3.0	0.49

data obtained from these experiments with the surface active agents listed in order of increasing duration of hypnosis. Igepal CA and the agents listed below it in the table gave a significant increase in the time of hypnosis.

As an attempt to test the mechanism of action with thiopental sodium some mice were sacrificed and analyzed at various intervals for the content of thiopental in the body (Table IV). The mice were injected into the tail vein with thiopental sodium, with and without Aresklene 400, 50 mg per kg. Each mouse was placed in a jar and at 30 minutes or 2 hours later killed and ground in this same jar with chloroform and buffer added. The analyses were carried out according to a modified method of Jailer and Goldbaum(7,

ty mice per group, 10 males and 10 females, received 300 mg per kg of sodium barbitol by tail vein. The controls received only sodium barbitol and the experimental animals received in addition Aresklene 400, 100 mg per kg, mixed with the barbitol sodium. The time without righting reflex was very variable in both groups, and there was probably no difference between the two groups. However, the average time for onset of hypnosis was 40.5 minutes after the administration of barbitol sodium alone, while with Aresklene 400 the average time of onset of hypnosis was 14.2 minutes. In a similar experiment on 20 rats with a dose of 200 mg per kg of barbitol sodium and Aresklene 400, 50 mg per kg, there was no difference either in the duration of hypnosis or in the time from injection until onset of hypnosis between the controls and those receiving Aresklene. Ten male rabbits

were given barbital sodium, 160 mg per kg, and half of them received Aresklene 400, 50 mg per kg, 2 minutes before the barbiturate. The onset of hypnosis averaged 14.8 minutes for the controls and the average time with Aresklene 400 was 2.4 minutes. Five matched pairs of dogs were given barbital sodium, 140 mg per kg with one of each pair receiving in addition Aresklene 400, 25 mg per kg, 1 minute before the barbiturate. The time from an injection until the dog would not stand up was an average of 12.8 minutes for the controls and 2.0 minutes for those receiving Aresklene 400. Ten other surface active agents in doses of one-half the LD₅₀ were then tested for the effect on the onset of hypnosis in mice (Table V). Female mice were used for this experiment. Six of the agents showed a decrease in time of onset of hypnosis which was probably significant.

Summary. 1. The duration of hypnosis of

pentobarbital sodium was increased for mice by all 11 surface active agents tested.

2. Aresklene 400 was selected for study on other animals. It increased the time of hypnosis due to pentobarbital in rats and rabbits but not in dogs.

3. Thiopental sodium showed an increase in effect with some of the surface active agents but not all.

4. Aresklene 400 increased the activity of thiopental apparently by decreasing the rate of destruction of thiopental in the body.

5. The onset of hypnosis but not the duration of hypnosis was influenced by Aresklene 400 with sodium barbital. Aresklene 400 decreased the time of onset of hypnosis when given with barbital sodium in mice, rabbits, and dogs but not in rats. Six of 10 other agents studied only in mice had the same effect on onset of hypnosis.

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Serological Tests on Soluble Antigens from Mice Infected with *Cryptococcus neoformans* and *Sporotrichum schenckii*.^{*} (17742)

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The purpose of the present paper is to show that soluble antigens of the fungus agents occur in various tissues and body fluids of mice infected with *Cryptococcus neoformans* and *Sporotrichum schenckii*, and that these antigens are readily detectable by direct serological tests analogous to those that have been used for the detection of soluble antigens in various bacterial, rickettsial and virus infections.

Methods. Thirteen strains of *Cryptococcus neoformans*, 4 strains of *Sporotrichum schenckii*; and, for control purposes, a strain of *Blastomyces dermatitidis* were used. The *Cryptococcus* included a representative strain of each of the types (A, B, C) recently re-

ported by Evans(1), and also the Curtis strain which Benham(2) found to differ somewhat in agglutinating properties from other strains. The mice were infected by intraperitoneal injections, and the materials to be tested were obtained from animals that were obviously ill or that had recently died from the infection.

The antigens employed for serological precipitation tests were wash fluids from the peritoneal cavity and extracts made from the liver, spleen, testes and lungs. The peritoneal wash fluids were formalinized, and cleared by prolonged centrifugation. The organs were ground in mortars, suspended in 5 ml of salt solution, shaken for about 5 minutes in tightly stoppered test tubes, then heated 30 minutes

^{*} This investigation was aided by a grant from The Louis Livingston Seaman Fund of The New York Academy of Medicine and by a fund from the Estate of Marguerite S. Davis.

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2. Benham, R. W., *J. Infect. Dis.*, 1935, v57, 255.

at about 50°C and finally boiled for 10 minutes (preliminary experiments had proved that the reactive constituents were relatively heat stable). Centrifugation of the heated liver and spleen suspensions yielded clear supernatant fluids, but shaking treatments with chloroform were required for the lungs and testes.

All of the antigen solutions were tested, in a series of dilutions ranging from 1:5 to 1:1600, against at least one *Cryptococcus* and one *Sporotrichum* rabbit antiserum; in the case of the peritoneal washings and the liver extracts from the *Cryptococcus*-infected mice, 6 different *Cryptococcus* antisera (produced by immunization with separate strains) were used. All the antisera had relatively high homologous agglutinating titres and were used in 1:5 or 1:10 dilutions for the precipitation tests. For controls, antisera of *Candida albicans* and of R pneumococci and normal rabbit sera were included in all the tests.

Results. The peritoneal wash fluids and the tissue extracts from all the mice infected with *Cryptococcus neoformans* and with *Sporotrichum schenckii* gave precipitation with the antiserum of the homologous fungus but not with any of the other sera included in the tests. Additional evidence of the specificity of the reactions was furnished by the fact that similar materials from mice infected with *Blastomyces dermatitidis* and from normal mice failed to react with the *Cryptococcus* and the *Sporotrichum* antisera.

It is evident that the fungus antigens were present in considerable amount since positive reactions were obtained with 1:400 or higher dilutions of the solutions prepared from most of the mice infected with *Cryptococcus* and with 1:25 or 1:100 dilutions of the solutions from the mice infected with *Sporotrichum*. The precipitation was always apparent within 5 to 10 minutes at room temperature, although there was an increase in the intensity of the flocculation, and usually also in the maximum reacting dilution after incubation for 2 hours at 37°C plus icebox storage.

Soluble antigen was found also in the blood of *Cryptococcus*-infected mice. The animals were chloroformed and blood from the heart was citrated and centrifuged immediately.

The plasma gave precipitation, in dilutions ranging from 1:5 to 1:25, against *Cryptococcus* antisera and was non-reactive against control sera. Similar experiments were not made with *Sporotrichum*-infected mice.

In addition to the described precipitation tests, positive Quellung(3) reactions were obtained with the *Cryptococcus* and *Sporotrichum* cells contained in the sediments from the centrifuged peritoneal washings and in unheated samples of the tissue suspensions. The Quellung reactions made the capsules of both these fungi much more prominent and sharply defined, which facilitated the microscopic detection of the fungus cells and their differentiation from blood and tissue cells. With some of the *Cryptococcus* preparations which contained especially large amounts of free soluble antigen, washing of the fungus cells was important in order to get prominent Quellung reactions.

Since all strains of *Cryptococcus neoformans* are not serologically identical(1,2), our experiments are not comprehensive enough to prove that the antigens of all strains of that species could be detected with a single antiserum. However, the materials from mice infected with all the strains we tried gave both precipitation and Quellung reactions with all our antisera, which had high homologous titres; the only difference observed was that lower dilutions of antiserum were required in the case of the materials from the mice infected with Evans'(1) type C strain.

Discussion. The present demonstration of soluble antigens in the tissues of mice infected with *Cryptococcus neoformans* and with *Sporotrichum schenckii*, is of academic interest because of the general lack of information on the presence and distribution of soluble antigens in hosts infected with mycotic agents.

Whether or not direct serological tests would be useful in the study of materials from human cases of cryptococcosis and of sporotrichosis can be determined only by trial in hospital laboratories. An application especially worthy of trial would be the test of spinal fluid from cases of suspected cryptococcal meningitis

3. Neill, J. M., Castillo, C. G., Smith, R. H., and Kapos, C. E., *J. Exp. Med.*, 1949, v89, 93.

(torulosis). With that material, one could try a combination of tests for serological precipitation upon the supernatant and of tests for Quelling reactions upon the sediment obtained by centrifugation of the spinal fluid, in a manner analogous to the procedures employed with spinal fluids from cases of pneumococcal, meningococcal, and *Hemophilus influenzae* infection.

It should be noted that antisera of reasonably high potency[†] are essential for the

optimum results both in precipitation of *Cryptococcus* soluble antigens and in Quelling of *Cryptococcus* capsules. Some cross-reactions occur between *Cryptococcus neoformans* and *Torulopsis rotundata*, various other encapsulated saprophytic yeasts, and type 2 pneumococci. These serological inter-relationships, which will be reported in a later paper, would have to be taken into account in the interpretation of precipitation reactions, but could readily be controlled.

Summary. The experiments showed that soluble antigens of the fungus agents occur in the tissues of mice infected with *Cryptococcus neoformans* and with *Sporotrichum schenckii*, and that these antigens are readily detectable by tests with appropriate antisera. Soluble antigen was found also in the blood of *Cryptococcus*-infected mice.

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Variations in Proteolytic Activity of Serum of Animals Including Man.* (17743)

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Under normal conditions the blood plasma or serum of man shows little spontaneous proteolytic or fibrinolytic activity, although Dastré(1) early indicated that such enzymes were present. However, as shown by Delezenne and Pozerski(2) and others(3,4), treatment

with chloroform produces definite proteolytic activity. Whether this be due to removal of inhibitor or true activation of the precursor has been a disputed subject. Yamakawa produced activity by use of other substances, including alcohols(5). The most consistent, definite and rapid activator has been streptokinase(6-8), but Gerheim *et al.* have also found an active staphylokinase(9). Grob(10) has

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reviewed the numerous materials which have been reported to result in activity. Milstone(6) was able to separate the proteolytic enzyme precursor which he called plasminogen and Tagnon *et al.*(4) separated the chloroform activated enzyme. Both workers indicated that it was present in the euglobulin fraction of serum. That the plasmin (activated plasminogen) is similar to trypsin in that it is a proteolytic enzyme which affects fibrin, fibrinogen, casein, gelatin, etc. has been proven(4,7). However, it has been shown that they are not identical because of a difference in the optimum pH for activity, quantitative difference in the breakdown of the above substances and tests on specific substrates(7,8). Most studies of the proteolytic action of plasmin have been made by testing its fibrinolytic activity. Increased fibrinolytic activity of the blood has been observed in shock(11,12) after the injection of adrenalin(13), at postmortem after death from severe trauma(14), with peptone shock in dogs(15), after electrically induced convulsions(16), and in liver disease(17,18).

A method for assay of the proteolytic precursor (plasminogen) was developed using a method for separation of plasminogen first suggested by Milstone(6), and a colorimetric estimation of tyrosine produced by digestion of casein. This method was described in detail, with data suggesting activation by protamine(19). Sera of 127 patients were ex-

amined, several on more than one occasion. Sera of 22 animals including 6 (*Macacus rhesus*) monkeys, 3 swine, 3 guinea pigs, 2 rabbits, 8 dogs and 1 goat were examined, and variations of the activation pattern between other animals and man were observed.

Results. In the previous report it was noted that with duplicate blood samples satisfactory results with a minimal experimental error were obtained (Table I). Using blood obtained from the same patient on separate days without significant change in their condition very similar results were obtained (Table II).

TABLE I.

Pt.	Sample	S.	P.	O.
M.	1	1.2	.39	.305
	2	1.16	.377	.295
G.	1	1.045	.225	.186
	2	1.08	.21	.197
H.	1	.535	.200	.173
	2	.52	.203	.186

Results obtained with duplicate samples of serum, in mg tyrosine produced. As in all tables: S = Streptokinase activation; P = Protamine activation; O = Spontaneous activation.

TABLE II.

Pt.	Date	Mg tyrosine produced by plasminogen activated by:		
		S.	P.	O.
L.R.	12/ 9/48	.836	.165	.055
	13	.858	.128	.033
J.E.	16	.150	.187	.045
	27	.123	.228	.092
J.N.	2/ 8/49	.274	—	—
	9	.275	.057	.015
M.L.	1/12	.385	.062	.088
	2/ 2	.388	.058	.05
M.B.	24	.706	.087	.035
	3/ 1	.680	.145	.075
B.G.	4	.991	.17	.123
	14	1.020	.188	.105
J.S.	5/ 3	.267	.023	—
	5	.300	.070	.006
	9	.320	.081	.024

Results in mg tyrosine produced obtained with separate samples of blood, drawn from one to 20 days apart. No significant changes in patient's general condition, but no attempt made to draw blood under identical conditions. S, P and O as in Table I.

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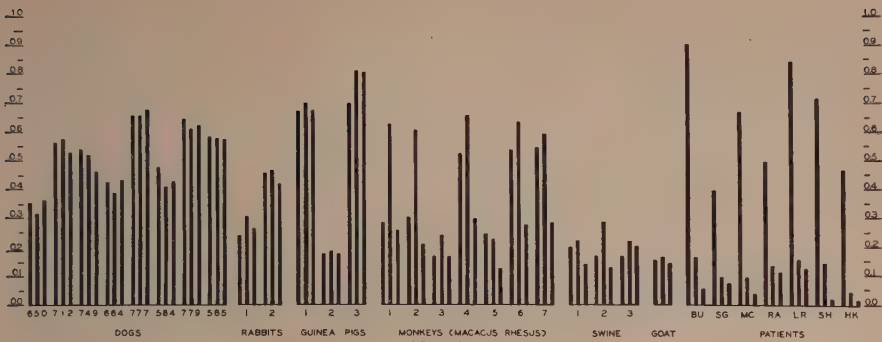


FIG. 1.

Columnar graph to show variations in amount of tyrosine produced by plasmin from animals of several species. A few representative normal men and women have been chosen and all other animals tested have been recorded. Most had repeated tests with similar results. For each individual animal the three columns are from left to right: (1) Streptokinase activator; (2) Protamine activator; (3) No activator (spontaneous). Other tests have been carried out on dogs and monkeys with similar results.

Sera from humans without disease and indeed with all disease except streptococcal infections[†] showed a characteristic activation pattern with streptokinase giving the most rapid and complete activation, protamine giving a definite activation and spontaneous activity being less than either in most instances (Fig. 1). Among the other species the activation pattern differed significantly, with the plasminogen from dogs, guinea pigs, rabbits, swine and one goat, giving almost and at times as much spontaneous activity as with the streptokinase or protamine activated material (Fig. 1). The plasminogen of the monkeys (*Macacus rhesus*) with one exception showed activation by streptokinase similar to that of the human, though to a lesser degree. Activation by protamine, however, was greater in the case of monkeys than with any other species tested. In six species studied, except man, the activity of the protamine-activated plasmin frequently exceeded that of the streptokinase-activated plasmin.

Since the data recorded on the chart were obtained, sera from 12 dogs, 3 monkeys and approximately 125 patients have been tested as a routine reaction by a technician with results essentially the same as those charted.

No explanation for this variation in activation pattern of the proteolytic precursor found in the euglobulin fraction of serum in various animal species is at hand. It is possible that

the struggling and anxiety instituted in the animals might possibly cause spontaneous activation, as has been shown to occur to a lesser degree with shock(11,12) and with injection of adrenalin into humans(13). It is conceivable that these animals have all had streptococcal infections and so have developed antibodies to streptokinase. However, their spontaneous activity is of the order of streptokinase activated plasmin in human sera (Fig. 1), which would make it appear we were testing their total activity. The most likely possibility is of course that the euglobulin material itself is significantly different in the different species. Previous study using the fibrinolytic method would tend to confirm this opinion, since variations in the fibrinolytic mechanism of streptokinase from organisms affecting different species have been observed(20,21,22,23,24). This variation is of some importance in the study of the proteolytic-antiproteolytic balance of the serum since much of the experimental work has been done on dogs, guinea pigs and rabbits, in which the mechanism of proteolytic activation

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is apparently different, and so correlation between these results and human reactions must be carefully analyzed. These data would suggest that monkeys should be used for animal experimentation on this complex system.

Summary. An euglobulin fraction of serum or plasma from animals and man contains a

proteolytic precursor or active enzyme. The activation pattern of this enzyme precursor in man and monkeys is different from that of dogs, guinea pigs and rabbits.

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Absence of Hemorrhage Inducing Activity in Phthiol Derivatives with a Basic Nitrogen in Side Chain. (17744)

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In previous reports(1,2) it was shown that 3-hydroxy-1,4-naphthoquinone derivatives, substituted in the 2 position with a hydrocarbon chain of 6 or more carbon atoms, induced a hemorrhagic syndrome in the rat. This syndrome, characterized by a marked hypoprothrombinemia, could be prevented by administration of vitamin K (2-methyl-1,4-naphthoquinone) or K₁ (2-methyl-3-phytyl-1,4-naphthoquinone), the latter being more active than the synthetic compound. From these findings it was postulated that these phthiol analogues competed with vitamin K in the processes concerned with prothrombin production.

More recently we have examined 3 phthiol derivatives* with a basic nitrogen atom in the side chain attached in the 2 position of the naphthoquinone. These derivatives were A 223, 2 - (piperidinomethyl) - 3 - hydroxy-1,4-naphthoquinone, A 1506, 2-(n-butylaminomethyl)-3-hydroxy-1,4-naphthoquinone, and A 1507, 2-(n-decylaminomethyl)-3-hydroxy-1,4-naphthoquinone.

The technics employed in the present study were the same as those used previously(1,2). Male rats, 4-5 weeks old, Sprague-Dawley strain, were used and were fed Purina Dog Chow checkers and tap water *ad libitum*. The

drugs, suspended in olive oil, were administered once daily via stomach tube in doses of 400, 800, and 1600 mg/kg. Six rats were given each drug level and the treatments were given for 10 days if the rats survived that long. Prothrombin time determinations(3) were performed on whole blood obtained by cardiac puncture either at the end of the treatment period or when a rat became moribund. At the end of the treatment period all rats were sacrificed and necropsies were performed.

Compound A 223 was found to be about 4 times as toxic as A 1506 and considerably more than 4 times as toxic as A 1507. Two of the 6 rats receiving 400 mg/kg A 223 succumbed; larger doses were uniformly fatal. A 1506 retarded growth at a dose of 800 mg/kg and was lethal to 4 of 6 rats at 1600 mg/kg. No toxicity other than a slight retardation of growth was noted with A 1507 even when doses of 3200 mg/kg were administered. The rats intoxicated with A 223 or A 1506 were usually pale and cyanotic. There was no evidence, however, of the hemorrhagic syndrome encountered in the previous studies. Prothrombin times of both the treated rats and the control animals were all within or close to the normal range (15-18 seconds). Occasionally in the rats receiving A 223 or A 1506 splenic enlargement and a brownish pigmentation of the lungs, heart, and skin were observed at necropsy. It was thought that this pigmen-

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* These compounds were obtained from Dr. M. T. Leffler, Abbott Laboratories.

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tion and the cyanosis might have resulted from intravascular hemolysis of red cells caused by the drugs. However, as shown by the results of hypotonic fragility studies, the compounds were not in themselves hemolytic.

It has been postulated(4) that the config-

uration $\begin{array}{c} \text{O} \quad \text{R} \quad \text{OH} \\ || \quad | \quad | \\ -\text{C}-\text{C}=\text{C}- \end{array}$, present in previously studied active naphthoquinone, hydroxycoumarin, and indandione(5) derivatives, is necessary for antivitamin K activity. The results obtained in this study show that in defining the moiety necessary for antivitamin K activity, at least in naphthoquinone derivatives, one must specify the nature of the sub-

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stituent R attached to the 2 position. Furthermore, the findings of Seeler(6) and Mushett(7) with 2-sulfanilamidoquinoxaline demonstrate that certain compounds with other configurations are active in antagonizing vitamin K.

Summary. Three phthiocol derivatives containing a side chain with a basic nitrogen have been examined for hemorrhagic activity and found to be inactive. The bearing of these findings on the structural configuration necessary for antivitamin K activity has been discussed.

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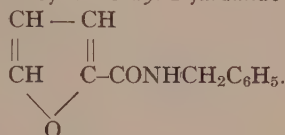
Action of N-Benzyl-2-furamide in Experimental Tuberculosis. Comparison with a Sulfone and Streptomycin. (17745)

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In a review of the chemotherapy of tuberculosis Rist(1) cites the work of Shpanir and associates(2) suggesting an antitubercular activity for the benzylamide of α furan carboxylic acid. The moderate activity of the sulfones in experimental tuberculosis and their ability to potentiate the action of streptomycin(3,4) prompted a search for other possible leads. Accordingly an experiment was set up to determine the potential antitubercular possibilities of this furan derivative.

Preparation of N-Benzyl-2-furamide (NBF)—



This compound, reported by Bowen and Smith(5), was synthesized by the action of furoyl chloride upon benzylamine in aqueous solution, with the addition of sodium hydroxide solution at intervals in order to neutralize the hydrochloric acid formed. The reaction was complete after 6 hours. After recrystallization from alcohol, the colorless crystals melted at 112-113°. This compound is only slightly soluble in water.

Toxicity. NBF was tolerated in guinea pigs in single doses of 0.5 g/kg when given orally

* N.I.H. Fellow.

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TABLE I.
Toxicity of N-Benzyl-2-furamide in Guinea Pigs. Oral Administration of Aqueous Suspension with Gum Acacia.

Dose, g/kg	No. used	No. died	Remarks
2.0	5	5	All died in 1-4 days. Labored respiration and progressive depression
1.5	5	2	Died in 1-3 days
1.0	5	1	Died in 3 days
0.5	5	0	

TABLE II.
Activity of N-Benzyl-2-furamide (NBF) in Experimental Guinea Pig Tuberculosis as Compared with That of a Sulfone (GLS) and Streptomycin (SM). All animals infected with 0.5 mg H37Rv Intraperitoneally.

Group	Treatment	No. used	Avg wt gain, g	No. died	T.B. index*		Chemotherapeutic effectiveness†
					Range	Avg	
1	Controls	9	77	5	8-20	14.4	
2	SM 20 mg/kg	10	285	0	±4	1.5	9.6
3	SM 40 mg/kg	10	259	0	0-2	0.9	16.0
4	NBF 250-500 mg/kg	9	34	7	5-19	12.2	1.2
5	GLS 100 mg/kg	10	230	1	1-11	5.0	2.9
6	SM 20 mg/kg + NBF 250-500 mg/kg	7	234	0	0-3	1.1	13.1
7	SM 20 mg/kg + GLS 100 mg/kg	6	220	0	0-2	0.7	20.5

* Extent of tuberculous involvement, rated as previously described. See Ref. 6.

† Average T.B. index of controls/treated.

in aqueous suspension with gum acacia. Larger doses killed some or all of the animals in 1-3 days as shown in Table I. Visceral congestion, mottling of the liver, pleural effusion and ascites were seen at autopsy. The compound was administered to the limits of tolerance, in daily doses of 250-500 mg/kg, in the therapeutic tests described below.

Antitubercular Activity. A series of male guinea pigs weighing about 300 g were inoculated intraperitoneally with 1 cc of a homogeneous suspension of tubercle bacilli containing 0.5 mg, moist weight of the human strain H37Rv. They were divided into 7 groups as follows:

1. 9 controls
2. 10 treated with 20 mg/kg streptomycin intramuscularly once daily
3. 10 treated with 40 mg/kg streptomycin as above
4. 9 treated with 250-500 mg/kg NBF orally once daily
5. 10 treated with 100 mg/kg galacturonic acid derivative of 4,4'-diaminodiphenyl-sulfone (GLS) orally once daily
6. 7 treated with 20 mg/kg streptomycin

intramuscularly and 250-500 mg/kg NBF orally, each once daily.

7. 6 treated with 20 mg/kg streptomycin intramuscularly and 100 mg/kg GLS orally each once daily.

All treatments were begun the day after infection and continued 5 days a week for 10 weeks. At the end of the experimental period all the survivors were killed with chloroform, autopsied and the extent of tuberculous involvement rated as previously described (6).

The results of this are summarized in Table II. The data indicate practically no activity for the furan derivative. The sulfone on the other hand, which was used for comparison, had a moderately good activity, almost one third that of streptomycin, at the dose levels employed. It should be mentioned that the activity of the sulfone in the present test was almost identical with that previously found (7).

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When used in combination with streptomycin the furan derivative showed only a slight degree of potentiation (chemotherapeutic effectiveness of 13.1 as against 9.6 ± 1.2 or 10.8), while the sulfone showed a high degree of potentiation. The chemotherapeutic effectiveness of combined therapy with streptomycin and the sulfone was 20.6 as against 9.6 ± 2.9 or 12.5, the sum of the effects of the two drugs. It should be noted that the chemotherapeutic effectiveness of sulfone-streptomycin combined therapy was somewhat greater than that of the double dose of streptomycin.

Comments and conclusions. The favorable results reported by Shpanir for the benzyl furamide were based on experiments in mice.

Though the mouse test is currently in use in several laboratories for screening of compounds for possible antitubercular activity we are not impressed with it as a satisfactory test object(8). It is possible that the present results are at variance with those of Shpanir on account of species differences.

N-Benzyl-2-furamide has no appreciable activity in experimental tuberculosis in guinea pigs. So far the sulfones alone, next to streptomycin, have been demonstrated to have a definitely suppressive effect on this disease.

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Oral Treatment of Pernicious Anemia with Swine Duodenal Mucosa and Vitamin B₁₂. (17746)

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Since the initial report by West(1) there has been ample evidence that the parenteral administration of as little as 1-2 μ g of vitamin B₁₂ daily to pernicious anemia patients in relapse is followed by reticulocytosis, rise in hemoglobin and erythrocytes, and clinical remission(2-6). The minimal effective oral daily dose lies between 75 and 150 μ g(7,8).

However, the oral administration of gastric juice from a normal person with 5 to 10 μ g of vitamin B₁₂ results in satisfactory hematologic and clinical response(9,10). More recently Bethell has shown that the ingestion of an extract of desiccated swine duodenal mucosa and 5 μ g of vitamin B₁₂ daily will induce a similar reaction(11). It is the purpose of this report to present additional data confirming this latter observation.

Two patients with typical Addisonian pernicious anemia in relapse were treated with daily oral doses of 2 g of an aqueous extract of swine duodenal mucosa and 10 μ g of vita-

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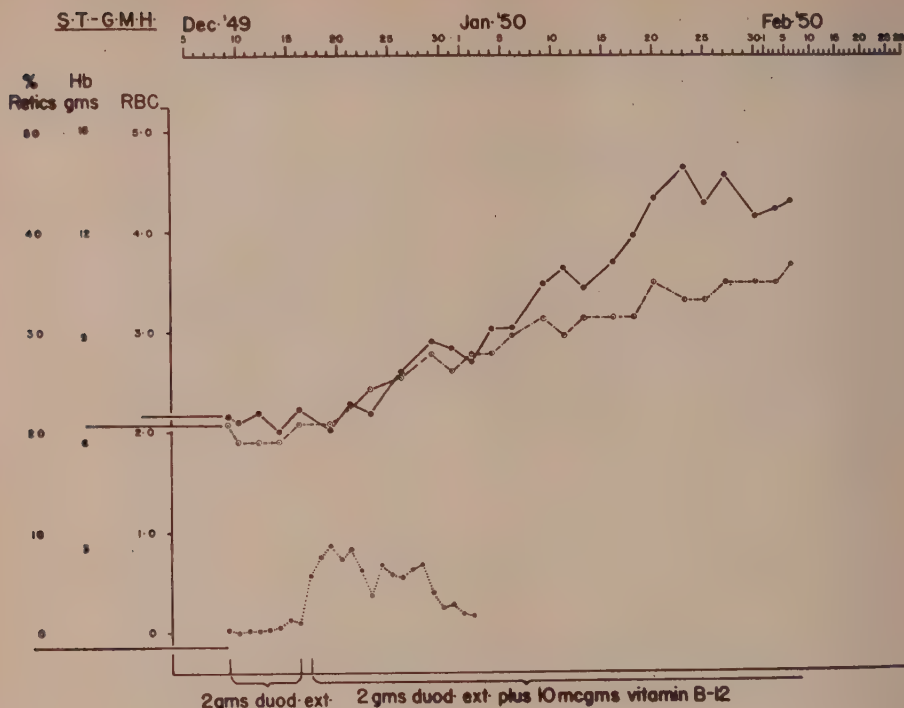


FIG. 1.

Hematologic data on patient, I.S., treated with oral daily doses of 2 g of swine duodenal mucosa and 10 μ g of vit. B₁₂.

min B₁₂ (Rubramin).^{*} The mucosal extract was given daily for one week before the vitamin B₁₂ was added in order to insure against any activity in the extract when administered alone. Previous studies have already demonstrated that daily oral doses of vitamin B₁₂ below 75 μ g are ineffective in the treatment of pernicious anemia (7,8). Both cases showed hyperchromic macrocytic anemia, histamine fast achlorhydria, and megaloblastic bone marrow. X-ray examinations of the gastro-intestinal tract, urinalyses and blood chemistry determinations were all normal. In one patient there was evidence of an old vascular accident of the left internal capsule. The other person was senile and a satisfactory evaluation of the neurologic status could not be made.

^{*} The materials used in this study were supplied by Dr. Geoffrey Rake of the Squibb Institute for Medical Research, New Brunswick, N.J.

I. S., male, age 83 years, was admitted to the hospital because of weakness and pallor. He was a known case of pernicious anemia treated years ago with ingestion of liver extract but stopped therapy of his own accord. At the time of the present admission he was completely disoriented so that a reliable history and neurologic survey was not possible. Physical examination disclosed marked pallor and an icteric tint. Liver was felt 2 cm below the right costal border. Knee and ankle jerks were sluggish. The initial blood count was hemoglobin 6.5 g, erythrocytes 2.15 million/cu mm and leukocytes 4450/cu mm. He received 2 g of desiccated duodenal mucosa extract daily for one week. The clinical condition deteriorated slightly and the hematologic status was unchanged. Ten μ g of vitamin B₁₂ were added to the mucosal extract and given orally, daily, thereafter. The hemoglobin, red blood cell and reticulocyte values are shown in Fig. 1. A suboptimal reticulocyte

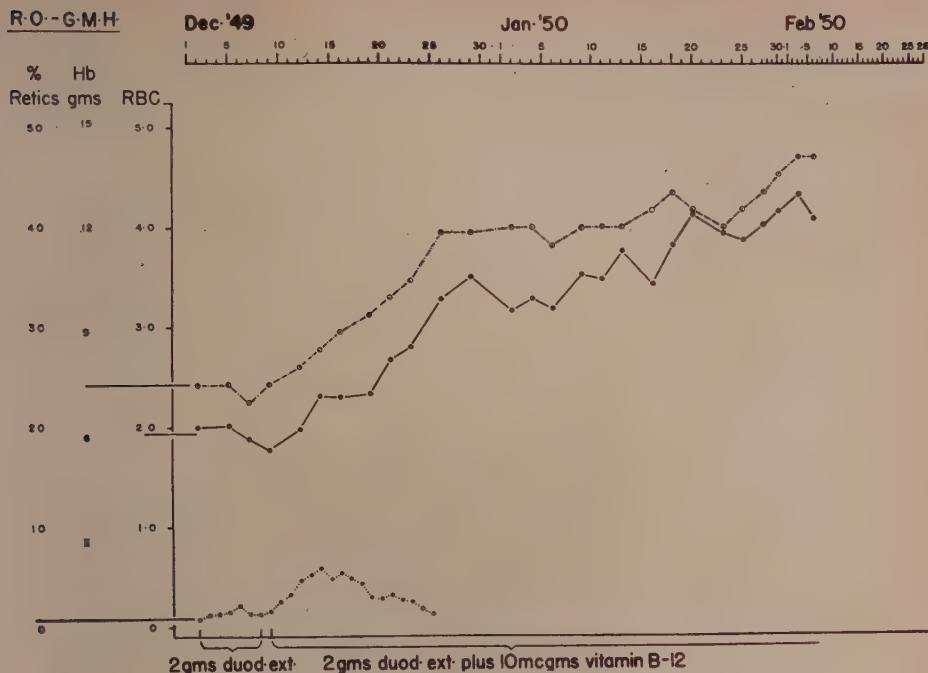


FIG. 2.

Hematologic data on patient, I.R., treated with oral daily doses of 2 g of swine duodenal mucosa and 10 μ g of vit. B₁₂.

response with a peak of 8.6% was observed on the 3rd to 5th days after the combined therapy was started. Clinical improvement was slow. The mental deterioration did not change. At the end of 8 weeks of treatment the hemoglobin reached 11.0 g and the erythrocytes 4.28 million per cu mm.

I. R., female, age 71 years, was admitted to the hospital because of weakness and spells of unconsciousness. She was observed at another institution for 3 months but no diagnosis was reached. Her appetite was poor and she lost 4 pounds during the past 2 months. She had difficulty in walking but never experienced any paresthesias. Physical examination disclosed the liver edge palpable at the costal margin. Slight pitting oedema of the legs was present. Positive neurologic findings were compatible with those following a vascular accident involving the left internal capsule. The initial hemoglobin was 7.5 g, erythrocytes 2.00 million/cu mm, and leukocytes 3800/cu mm. She was given 2 g of desiccated hog duodenum mucosa daily for one week with no

signs of hematologic change. Thereafter, 10 μ g of vitamin B₁₂ were added to this daily dose. A suboptimal reticulocyte response of 5.9% was observed on the 5th day (Fig. 2). Clinically there was slow subjective improvement. The hemoglobin rose to 13.5 g and erythrocytes to 4.15/cu mm after 2 months of treatment. The neurologic status remained unchanged.

Discussion. The nature of intrinsic factor which is present in normal human gastric juice and in the mucosa of the stomach and duodenum of swine is at present under investigation. Agren and Waldenstrom have shown that a highly purified amino polypeptidase isolated from the pyloric mucosa of swine, incubated with fresh liver, and administered to patients with pernicious anemia in relapse, induced a satisfactory hematologic and clinical remission(12). More recently it has been observed that the simultaneous oral administration of subminimal doses of pteroyl

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glutamic acid and vitamin B₁₂ to cases of pernicious anemia in relapse results in an excellent hematologic and clinical response(13).

Vit. B₁₂ and at least one other growth factor are present in the mucosa of the stomach of the pig(11,14). No attempt was made to determine the vit. B₁₂ activity of the extract used in the present study. However, since there was no response to the ingestion

of this material after one week, followed by hematologic and clinical improvement when it was combined with 10 µg of vit. B₁₂; it would appear that there was not enough present for therapeutic efficacy when administered alone.

Conclusion. The oral administration of an extract of swine duodenal mucosa and subminimal doses of vitamin B₁₂ to patients with pernicious anemia in relapse is followed by a suboptimal reticulocyte response and satisfactory hematologic and clinical improvement.

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Experimental Pulmonary Edema. III. Hypoglycemia, a Cause of Pulmonary Edema.* (17747)

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During a study of long continued hypoglycemia in the rat(1) it was found that death from respiratory failure always ensued and there was marked edema of the lungs. Some time before death the pulmonary pathology was evidenced by the oozing of blood-tinged serous fluid from the nostrils of the comatose animals. This recalled the edema of rats after an overdose of epinephrine(2,3) and the possibility occurred to us that the effect of insulin was, at least in part, an overproduction of epinephrine resulting from the hypoglycemia. At the time we were able to demonstrate measurable concentrations of epinephrine by bioassay in the blood of rats with insulin shock. Removal of the adrenal medullas with sufficient time allowed for regeneration of the cortical tissue prior to insulin treatment failed to prevent the development of the pulmonary pathology in most rats. We have now studied the hypoglycemia lung edema further with special reference to the effect on it of an adrenergic blocking agent.

Experimental. The rat (Slonaker strain(4)) groups were made up of equal numbers of both sexes within a weight range of 40 g. To produce a hypoglycemia the rats were fasted for 24 hours and given 2 units of regular and 2 units of protamine insulin (u-40)[†] per 100 g body weight by subcutaneous injection. One group had been adrenalectomized the preceding day and another group had their adrenals demedullated the week before. After they succumbed to the hypoglycemia the lungs were removed and weighed. Our data are summarized in Table I.

Discussion. Prior adrenalectomy (Grp. 2) reduced the degree of pulmonary edema as measured both by inspection and lung weight but this may have been due to the naturally poor condition of the animals when tested. However demedullation of the adrenals (Grp. 3) from which the animals had completely recovered reduced the degree of edema of the lung just as much. It seems probable then that hypersecretion of the adrenal medulla due to hypoglycemia definitely contributes to the occurrence and extent of pulmonary

* This study was supported by a grant from the Life Insurance Medical Research Fund.

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[†] Kindly supplied by Dr. F. B. Peck of Eli Lilly and Co.

TABLE I.

Group No.	No. rats	Avg body wt, g	No. rats with lung edema	Avg degree of edema	Lung wt per 100 g body wt, g	% control
1	24	116	0	0	.60 $\pm$.05	100
2	28	114	27	2.1	1.29 $\pm$.14	215
3	24	119	1	0	.57 $\pm$.03	95
4	8	155	6	1.0	.84 $\pm$.09	140
5	16	152	14	1.6	.94 $\pm$.11	158

edema. The adrenergic blocking agent, N-(9-fluorenyl) - N-ethyl- β -chlorethylamine-HCl,[†] for practical purposes prevented the hypoglycemia pulmonary edema entirely. It was administered as described before(5).

What then is the mechanism of the hypoglycemia lung edema described here? The chief theories as to the mechanism of pulmonary edema have recently been briefly reviewed by Paine and Smith(6) and in more detail by others(7,8). In short the leading theories are that it is due to left ventricular failure in relation to right heart action on one hand or neurogenic due to intense stimulation of the vasomotor nerves to the lungs either directly or as a result of reflexes arising in the various viscera, the heart, etc. Visscher's group(9) does not separate these as two mechanisms. Their hypothesis, in the case of the pulmonary edema associated with increased intracranial pressure, is that brain compression is associated with increased vagal tone, and the latter results in bradycardia, lowered cardiac

output, high pulmonary vascular pressures and lung edema.

It is unlikely that hypoglycemia produces left ventricular failure directly. The greatly lowered blood sugar could easily impair the nutrition of the cardiac musculature but if this were the cause of the edema an adrenergic blocking agent could hardly prevent it. Much more likely is that a volley of impulses come down the sympathetic innervation of the lungs (and heart?) as a result of the stimulation of the autonomic system and medullary brain centers known to be afforded by an insufficient supply of glucose(10). The adrenergic blocking agent used presumably prevents these stimuli from reaching the lungs (and heart?).

Summary. Albino rats of the Slonaker Strain when kept in a state of hypoglycemia by insulin injections for a period of some hours invariably develop a fatal pulmonary edema. Adrenalectomy and adrenal demedullation reduce the degree of edema but fail to prevent it. Adequate doses of the adrenergic blocking agent, N-(9-fluorenyl)-N-ethyl- β -chlorethylamine-HCl, prevent this hypoglycemia lung edema entirely. -It is suggested that this type of pulmonary edema is neurogenic in origin due to the influence of glucose lack on the nerve centers where the vasomotor fibers to the lungs and heart originate.

[†] We are indebted to Dr. Glen E. Ulyot of the Smith, Kline and French Laboratories for supplies of this drug under the code name of "SKF-501."

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In Vitro Sulfate Fixation by Granulation Tissue and Injured Muscle Tissue from Healing Wounds.* (17748)

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It has been reported by Singher and Marinelli(1) and confirmed by Dziewiatkowski(2) that sulfate injected into the intact rat is concentrated *in vivo* in the cartilage, bone marrow and bone matrix of this animal. Some of our quantitative work indicates that this may not be the case for bone marrow free of bony and calcified material(3). By adapting the assay methods used by the workers cited to the conditions met with *in vitro* tissue work, the author(4) has shown that human normal and cancerous tissues maintained *in vitro* will exhibit qualitative differential fixation of the sulfate as insoluble tissue sulfate. This finding has been applied to the diagnosis of chondrogenic tumors. By removing muscle tissue from chick embryos at various stages in their development, and maintaining these tissues in a medium containing sodium sulfate labeled with radioactive sulfur, it was found(3) that embryonic heart, skeletal muscle, and other tissues absorbed sulfate quantitatively from the medium, and fixed the sulfate in the tissues as insoluble organic sulfate. The sulfate fixation capacity of embryonic skeletal muscle reached a maximum value of 1.5 μg of sulfate

ion per 100 mg wet tissue between the fourteenth and sixteenth days of embryonic development. After the sixteenth day of the sulfate fixing capacity dropped rapidly, becoming relatively insignificant (*i.e.* less than 0.10 μg per 100 mg tissue) one week or more after hatching.

That the sulfate was bound by the tissue, probably as organic sulfate, is indicated by the fact(3,4) that thorough washing with water did not remove the labeled sulfate absorbed from the medium by the tissues maintained at 38°–39°C. The labeled sulfate was entirely removed from tissues maintained at 0°C. Sulfate fixation is dependent upon the survival of the cells since it was shown that no fixation occurred when KCN was added to the medium. When the tissues were placed in the labeled medium and then immediately heated to 100°C and then cooled before incubation, no sulfate was fixed. Freezing the tissue with dry ice before incubating also destroyed its capacity to fix sulfate. Accidental contamination with bacteria reduced sulfate fixation by the tissues to very low values.

Since most tissues from rapidly growing embryos showed relatively high sulfate fixation values, as compared to muscle tissue from young and adult animals which showed almost no significant sulfate affinity, it was thought that the regenerating tissue from a healing wound in the adult animal might show an affinity for this ion similar to that exhibited by embryonic muscle at some stage in its development. With this in mind a preliminary experiment was conducted on a 4-month-old hen.

It was found that granulation tissue from wounds inflicted from 4 to 11 days previously showed *in vitro* sulfate fixing capacity equal to that of the corresponding muscle (*M. Tensor Fascia Latæ*) from the embryonic chick

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The investigation was initiated under a grant from the Nutrition Foundation, Inc., for the study of *in vitro* nutrition of tissues.

The radioactive sulfur employed was obtained from the Oak Ridge National Laboratory on allocation by the United States Atomic Energy Commission for research in cancer and growth.

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TABLE I.

Showing the Weight of Sulfate Ion Absorbed from the Medium and Fixed as Insoluble Tissue Sulfate by Tissue Fragments from Various Parts of Healing Wounds. Control samples are from the surrounding normal (unwounded) tissue of the same muscle 5 to 10 mm from the cut edge of the wound.

Chicken No.	Age when wounded, days	"Age" of wound, days	No. of samples	Description of tissue in sample from wound	μg of sulfate ion fixed per 100 mg wet tissue	
					Healing wound tissue	Original tissue from muscle (controls)
1	120	4	3	Pink granulation from center of wound	.70	.06
2	30	7	3	"	1.0	.00
3	30	7	2	"	.90	.03
4	32	4	3	"	.84	.00
5	32	4	3	"	.90	.00
6	32	4	1	"	.97	.06
3	32	4	1	Cut edge of healing wound	.97	—
2	40	6	1	Muscle near but not including cut edge of wound	.25	.04
3	40	6	1	New muscle fascia	.72	.00
4	42	6	3	Greyish granulation tissue from cut edge of wound	1.2	.03
4	42	6	3	Center of new pink hued tissue	1.1	—
4	42	4	1	Edge of wound (tissue grey with fine red spots)	1.26	—
4	42	fresh	2	Fresh blood clots	.04	—
1	130	6	3	Cut edge of wound	1.1	.00
2	44	4	3	"	1.1	—
3	44	4	1	New tissue from adhesion	1.43	—
3	44	4	3	Cut edge of wound	.80	—
4	46	4	1	"	1.96	.06
5	46	4	1	Granular tissue from surface of wound	2.27	.00
5	46	fresh	2	Fresh blood clots	.04	—
4	52	5	3	Pink tissue from center of wound	.80	—
4	52	5	3	New fascia	.44	—
5	52	5	5	Cut edge of healing wound	.94	—

on the seventeenth day of development (*i.e.* approximately $1.0 \mu\text{g}$ per 100 mg wet tissue). Samples of tissue removed at the time of the initial wounding showed no sulfate absorption, nor did tissue removed from the muscle 5-10 mm from the healing wound. Scar tissue removed from completely healed wounds showed no affinity for sulfate ion.

A series of experiments involving chicks approximately 30 to 50 days of age and one approximately 120 days of age was then conducted. The chickens were wounded by partial section of *M. Tensor Fasciae Latae* of both legs. Samples of tissue were removed from the wounds and from the surrounding tissue at irregular intervals over a period of one week, and new wounds inflicted at the time of tissue removal. The excised tissues were weighed, rinsed in the medium, and placed in 2 ml of the author's "X" medium.[†] After 40 hours in the incubator at 38.5°C the tissues were removed and the amount of sulfate fixed was determined by a method described in detail in another paper(3). Upon inspection of the data presented in the table, it will be seen that new tissue in the growth stages, and the recovering original tissue from the cut edge of the healing wound both show sulfate fixation values several times as great as for freshly excised, unwounded tissue from the same muscle. This relationship is similar to that of growing embryonic muscle tissue to

tissue from the corresponding muscle in the chicken several weeks after hatching(3).

In the publication cited(3) the author has suggested that sulfate is required for tissue formation in the embryo, probably being bound in the newly forming connective tissue substance. The fact that sulfate is absorbed and fixed in the regenerating tissue of muscle wounds of young and adult animals is further evidence of the utilization of sulfate in the anabolic processes involved in the synthesis of tissue substance. The fact that high sulfate fixing capacity is exhibited by the recovering original injured tissue at the edges of the new tissue might indicate that the processes involved in outgrowth of fibroblast cells from the original tissue, or the process of repair to the injured original tissue involves the absorption and fixation of sulfate in this tissue. It is possible that sulfate binding capacity may be a limiting factor in the rate of healing or replacement of connective tissue substance.

Experiments are now being conducted with a view to the elucidation of the mechanism of sulfate fixation, and toward the determination of the form in which sulfate is bound in the tissue.

Summary. Granulation tissue and injured muscle tissue from healing wounds were maintained *in vitro* in a medium containing inorganic sulfate labeled with radioactive sulfur, S^{35} . It was found that these regenerating tissues absorbed inorganic sulfate and fixed it in the tissues in an insoluble form. Uninjured muscle tissue from the same animals showed no significant sulfate fixing capacity. It is suggested that sulfate is utilized in the synthesis of the intercellular connective tissue substance, and hence is necessary to wound healing.

[†] Tyrode's solution modified to contain 4.8 mg of sulfate ion (as Na_2SO_4) per l. The sulfate was labeled with radioactive sulfur, S^{35} , to give a specific activity of approximately 107 counts per mg of sulfate ion, corrected for self absorption to 30 mg of BaSO_4 , and determined with a Geiger-Muller counter. Sulfate counting standards(3) were prepared from the culture medium in order that counting data could be translated into micrograms of sulfate fixed per 100 mg of wet tissue.

Liver Xanthine Oxidase and Q_{O_2} in Relation to Thyroid Activity* (17749)

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The effect of thyroidal activity on liver xanthine oxidase and liver Q_{O_2} was studied for several reasons. Brophy and McEachern(1) reported that the Q_{O_2} of rat liver slices is highly variable in hyperthyroidism, sometimes being abnormally low. Since the rate of oxygen consumption by a rat liver homogenate is dependent upon the xanthine oxidase activity of the liver(2), it seemed possible that the hyperthyroidal depression of the liver Q_{O_2} resulted from a loss of this enzyme. However, the results have shown that the Q_{O_2} of a liver slice, in contrast to an homogenate, is relatively independent of the xanthine oxidase activity of the liver. We have been unable to produce livers with low Q_{O_2} by the administration of thyroxine, but if this can be done in certain rats, it would seem to be unrelated to the liver xanthine oxidase.

The toxic effect of excess thyroxine in limiting the growth rate of young rats on a purified diet is an induced nutritional deficiency that can be prevented by feeding an unidentified factor found in liver(3,4). Normal levels of liver xanthine oxidase are also dependent upon a similar factor from liver(5). The possibility that the administration of thyroxine to rats on a purified diet would alter the liver xanthine oxidase was therefore investigated.

Experimental. Liver Q_{O_2} vs Xanthine Oxidase. The xanthine oxidase activity of

each liver homogenate was determined by the method of Axelrod and Elvehjem(2,6). The Q_{O_2} of the liver slice was measured in a similar setup, using oxygen as the gas phase and determining dry weights on alternate slices. Both determinations were made simultaneously on the same liver. Varying levels of liver xanthine oxidase were obtained by feeding adult and immature rats different diets. High normal levels were observed in rats maintained on Purina dog chow, while intermediate and low levels were obtained by feeding young rats a purified 21% casein or 8% casein diet(5) respectively for 1 to 4 weeks. All of the results have been plotted in a scatter diagram, Fig. 1, and show that the Q_{O_2} of the slice was unaffected by the xanthine oxidase activity of the liver unless the latter had been decreased to "zero" levels. The liver Q_{O_2} for 39 rats maintained on chow averaged 6.22 with a standard error of the mean of ± 0.11 ; the corresponding xanthine oxidase activity was 1377 ± 66 units. When the liver xanthine oxidase was depleted to "zero" levels (actually 50-100 units(2)), the Q_{O_2} of the slice was decreased by 17%, the average for 17 rats being 5.28 ± 0.25 ($P = < 0.01$).

The results show that the level of liver xanthine oxidase is not an important factor in determining the Q_{O_2} of the slice. The metabolic reactions occurring in a slice consume oxygen at a faster and more constant rate than in an homogenate, possibly due to the greater stability of the catalytic nucleotides in the slice. Tipping in xanthine to an actively respiring liver slice of normal xanthine oxidase activity increased the rate of oxygen consumption very little. Glycogen and glucose also did not appear to be substrates for the respiration of a slice, since the Q_{O_2} was unaffected by a 24-hour fast or by

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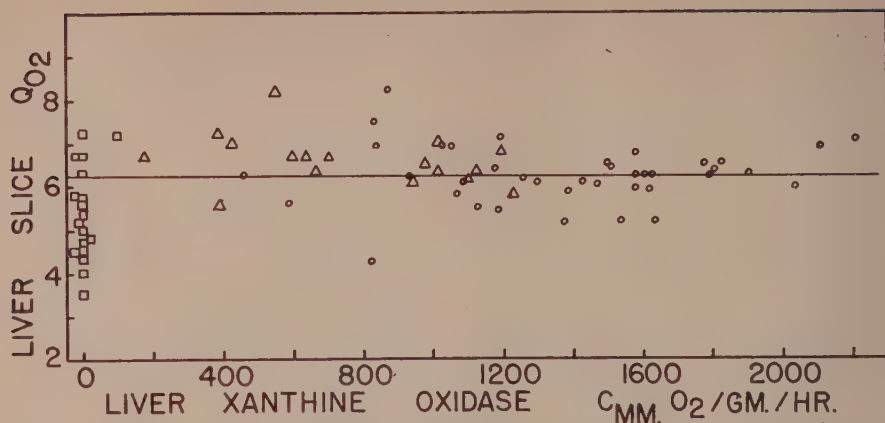


FIG. 1.

Scatter diagram showing the relationship between the Q_{O_2} of a liver slice and the xanthine oxidase activity of the liver. Varying levels of liver xanthine oxidase were obtained by feeding rats different diets: O = chow; Δ = purified 21% casein; $\square$ = purified 8% casein.

the addition of 100 mg glucose to the Warburg flask containing slices from either normal or fasted rats.

Thyroxine vs. Liver Xanthine Oxidase and Q_{O_2} . Thyroxine was administered to adult Sprague-Dawley male rats and to young albino female rats maintained on chow post-weaning. From 0.5 to 1.4 mg thyroxine was injected subcutaneously daily, or desiccated thyroid was added to the diet at a level of 0.5% until the rats were sacrificed for analysis of the liver. Rats receiving thyroxine were simultaneously fed chow, the 21% purified casein diet or the 8% casein diet. The low casein diet was fed for 5 days before the thyroxine administration was started while the 21% casein diet was started simultaneously with the thyroxine. 0.3% Thiouracil was also fed in a chow diet.

The results are shown in Table I. The known effect of thyroxine in significantly increasing the liver Q_{O_2} was observed on all 3 diets; in no instance did a low Q_{O_2} result from thyroxine administration. Thiouracil had a smaller but significant effect in decreasing the Q_{O_2} . The previously demonstrated effect of diet on liver xanthine oxidase(5) was again apparent in a comparison of the control groups on each diet. Since the young rats used in these experiments were maintained on chow for 2 to 3 weeks post-weaning, the liver xanthine oxidase was approximately 1500 to 1600

units at the start of the thyroxine studies(5). These levels were maintained in the chow-fed animals, but were decreased to 1100 - 1200 units by the purified 21% casein diet and to almost zero levels by the 8% casein diet. Adult rats showed little effect from the short period on the purified 21% casein diet.

Thyroxine depressed the growth rate approximately 50% in young rats on the purified 21% casein diet, but had no effect on the liver xanthine oxidase. When administered to adult male rats fed chow, thyroxine tended to increase the liver xanthine oxidase. In two additional experiments not recorded in the table, adult male rats fed 1% desiccated thyroid on chow for 11-13 days had average liver xanthine oxidase levels higher than the simultaneous chow fed controls by 48% ($P = <0.01$) and 20% ($P = 0.1$). When administered to rats receiving the low protein diet, thyroxine prevented the marked depletion of liver xanthine oxidase that was observed in the control group. In this respect thyroxine altered the response of liver xanthine oxidase from that characteristic of a low protein diet to an effect characteristic of a starvation regime, inasmuch as this enzyme can be removed almost completely from the liver by a low protein diet but can be decreased only about 50% by starvation(7).

Other Tests. Adult male albino rats main-

TABLE I.
The Effect of Thyroxine on Liver Xanthine Oxidase and QO₂ When Administered to Rats on Different Diets.

Exp. No.	Sex	Diet	Treatment		Duration, days	No. of rats	Body wt, g		Liver X.O.		Liver QO ₂	
			Type				Start	End	Mean ± S.E.	P	Mean ± S.E.	P
1	F	Dog chow	.5% dess. thyroid .3% thiouracil	—	—	4	114	145	1873 ± 128	—	6.09 ± .37	—
				22	20	4	95	119	1853 ± 131	1.0	9.44 ± .29	<.01
2	M	Dog chow	.5% dess. thyroid .3% thiouracil	—	—	6	365	352	1587 ± 251	0.3	5.21 ± .20	.07
				20	20	2	347	350	1536 ± 120	—	6.01 ± .12	—
3	F	21% casein	.5% dess. thyroid .3% thiouracil	—	—	6	91	112	2290 ± 30	0.07	7.72 ± .39	.02
				21	40	6	93	139	1224 ± 98	—	5.07 ± .30	—
				19	20	6	91	112	1096 ± 46	0.3	6.27 ± .14	<.01
4	F	21% casein	.5 mg thyroxine 1.0 mg thyroxine	—	—	7	105	133	1178 ± 55	0.3	9.99 ± .29	<.01
				14	15	9	108	118	960 ± 23	0.4	9.49 ± .39	<.01
5	M	21% casein	1.0% dess. thyroid	—	—	10	332	379	1241 ± 61	—	—	—
				9-30	12	12	320	246	1159 ± 66	—	6.32 ± .10	—
6	F	8% casein	1.4 mg thyroxine	—	—	8	107	110	1468 ± 103	1.0	8.75 ± .25	<.01
				8-13	9	12	107	95	1463 ± 81	<0.01	5.67 ± .39	—
				8-13	8	12	107	95	12 ± 12	<0.01	9.60 ± .20	<.01

TABLE II.
Effect of Growth Hormone and ACTH on Liver Xanthine Oxidase.

Exp. No.	Diet	Treatment	No. of rats	Body wt, g		Adrenal wt mg/100 g body wt	Liver xanthine oxidase	
				Start	End		Mean ± S.E.	
1	Dog chow	—	9	222	256	11.3	1866 ± 125	
		Growth hormone	9	231	273	13.1	1604 ± 81	
		ACTH	9	223	243	14.2	1888 ± 74	
2	Horse meat	—	9	227	274	12.8	2162 ± 125	
		ACTH	9	224	265	16.5	2176 ± 60	
3	Dog chow	—	9	255	262	—	1402 ± 96	
		Growth hormone	9	255	284	—	1466 ± 42	

tained on chow or horse meat were injected twice daily with Armour's[‡] growth hormone or ACTH. A total of 1 mg per rat per day was given for 14 days, after which the rats were sacrificed for a determination of liver xanthine oxidase. The results are shown in the first two experiments of Table II. Neither the growth hormone nor the ACTH had any effect on liver xanthine oxidase. The endogenous respiration of the livers from the rats treated with growth hormone or ACTH was the same as that for the controls. Horse meat gave significantly higher levels of activity than chow. The growth hormone experiment was repeated in female albino rats, and the results, Expt. 3 of Table II, again showed no effect. Methylene blue added to the Warburg flasks gave the same average 1.54 increase in xanthine oxidase activity in the rats treated with growth hormone as was obtained in the controls.

[‡] We are indebted to Dr. I. M. Bunting of Armour and Co., for the ACTH and growth hormone.

Summary. The Q_{O_2} of a liver slice, in contrast to an homogenate, was relatively independent of the xanthine oxidase activity of the liver, since the latter could be varied widely by dietary procedures without affecting the former.

Thyroxine increased the liver slice Q_{O_2} of rats fed stock chow, a purified 21% casein diet, or a purified 8% casein diet; no hyperthyroidal exhaustion of the liver Q_{O_2} could be produced.

Thyroxine had no effect on liver xanthine oxidase when rats were fed a purified 21% casein diet, but tended to give higher values with chow-fed adult rats. When administered with a low protein diet, thyroxine prevented the marked depletion of liver xanthine oxidase and gave results more characteristic of starvation than of a low protein diet.

Growth hormone and ACTH had no effect on liver xanthine oxidase.

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Effect of Particle Size of Suspensions of Estrogen Crystals on Duration of Estrus. (17750)

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The implantation of pellets of all hormonal steroids has become a common practice both in the laboratory and in the clinic since the demonstration by Deanesly and Parkes(1,2) that dry crystals or compressed pellets of androgens and estrogens when implanted, acted like a reservoir of the steroid and produced a prolonged physiological effect. Between solutions of steroids in water or oil and the pellets weighing several milligrams, lies a region of particle size which has been little investigated in a systematic manner. Richards, Spruth and Russell(3) showed that a single injection of

500 μ g (5,000 I. U.) of an aqueous suspension of small crystals of estrone that would pass a 26 gauge needle, resulting in an average duration of estrus 15-23.5 days in four different groups of castrate rats. The same amount of estrone in oil solution resulted in estrus lasting only 2 days. Recently this report has been confirmed by Simond *et al.*(4) but only high dosage levels of 5,000 to 20,000 I.U. were used.

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Miescher, Gasche and Frey(5) have studied the duration of effectiveness of crystalline suspensions of differently sized particles of α estradiol, α estradiol dipropionate, α estradiol monobenzoate and estrone but used only the one dosage level of 500 mcg. They also carried out some studies with progesterone. Meier, Gasche and Frey(6) conducted similar studies using desoxycorticosterone and found the duration of effectiveness to be proportional to dosage up to a single injection of 40 mg. They found crystalline suspensions to be about 10 times as effective as oil solutions. Prolongation of estrogenic activity in rats is also well established for certain esters of estradiol even though administered in oil solution(7). Because the clinical use of aqueous suspensions of estrogens has become extensive, further experimental studies on the quantitative aspects of dosage and particle size seem to be indicated.

We have studied the effect of variable amounts of estrone of certain particle sizes on duration of physiological activity using both estrus in adult castrate rats and uterine weight in castrate immature female rats as indicators.

Materials and methods. The estrogen used was a crystalline ketonic preparation from pregnant mares' urine consisting mainly of estrone. The crystals were screened through standard 60, 100, 200 and 325 mesh screens. Particles that passed through a 60 mesh screen and not through a 100 mesh screen were designated as 60 mesh size. Those which passed through a 100 mesh and not a 200 mesh screen were designated as 100 mesh size, and so on. A corn oil solution of the same crystalline estrogen was also prepared. The crystals were suspended in a medium containing 0.5% sodium carboxymethylcellulose (CMC)* and 0.125% polyoxyalkylene sorbitan oleate (Tween-80).† The CMC makes the solutions sufficiently viscous to prevent rapid settling

of the crystals. The Tween-80 helps to wet the steroid crystals so that they suspend readily. Adult ovariectomized rats, which had been primed with estrogens, and which were in condition to use for a routine estrogen assay were used in most of the tests.

The experimental procedure was to give subcutaneously, using a 22 gauge needle, a single injection of 0.1 to 0.5 ml of the aqueous estrogen suspension under test. Vaginal smears were taken, except on Saturday and Sunday. In order to provide continuous figures on the chart, half of each group was started on a Friday and the other half on a Monday so that all days would be covered by a portion of the rats. As a result, 4 days out of each 7 are represented by only half as many rats as the other 3 days. This gives rise to an increase in percentage estrus at some points along the curve. This increase is due to the use of averaged values from the two different groups and is not due to some of the rats going out of estrus and then coming back into estrus again. One series of experiments was carried out using rats ovariectomized when they were 21 days of age. After a recovery period of 2 weeks, these animals were injected without priming. Smears were taken at intervals. Groups of 4 rats were killed and the uteri excised and weighed.

Experimental. Duration of Estrus with Oil Solutions of Estrone. The cellular changes in the vagina which produce what is known as an estrus smear, require a period of about 36 hours to develop even with high dosage of estrogens. Hence, no rats were in estrus one day after injection. Using standard bioassay procedures, an oil or aqueous solution containing about 15 I.U. of estrone will produce estrus in 50% of a group of adult castrate rats about 36-48 hours after injection. Approximately 50 I.U. (5 mcg) will produce estrus in 100% of the animals. The duration of estrus in both cases is only about 24 hours or until 3 days after the initial injection.

As a basis for comparison between oil solutions and suspensions there are plotted in Fig. 2 and 3 the averaged data obtained with several groups of rats when given a single injection of an oil solution containing 10,000 I.U. (1 mg) of the crystals. About

5. Miescher, K., Gasche, P., and Frey, H., *Helv. Physiol. Pharm. Acta*, 1944, v2, 515.

6. Meier, R., Gasche, P., and Frey, H., *Schweiz. Med. Woch.*, 1946, v76, 107.

7. Pedersen-Bjergaard, K., and Tonnesen, M., *Acta Endocrinologica*, 1948, v1, 350.

* Hercules Powder Co., Wilmington, Del.

† Atlas Powder Co., Wilmington, Del.

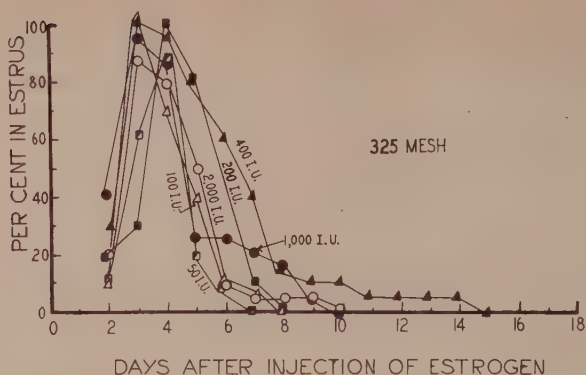


FIG. 1.

Duration of estrus in adult ovariectomized rats as a function of the dosage of estrogens. A single injection of an aqueous suspension of crystals of 325 mesh size was given. 20 rats were used on each dosage level.

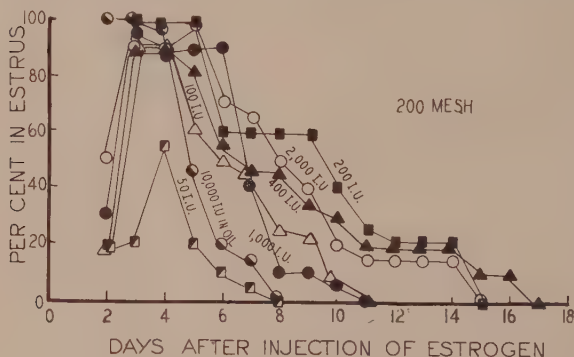


FIG. 2.

Duration of estrus in adult ovariectomized rats as a function of the dosage of estrogens. A single injection of an aqueous suspension of crystals of 200 mesh size was given. 20 rats were used on each dosage level.

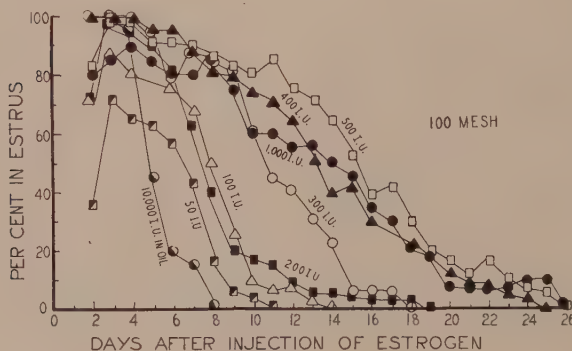


FIG. 3.

Duration of estrus in adult ovariectomized rats as a function of the dosage of estrogens. A single injection of an aqueous suspension of crystals of 100 mesh size was given. 60 rats were used on each dosage level.

50% of the rats were still in estrus on the fifth day, or for a period of time which is only two days longer than with 50 I.U. in oil.

Effect of Variable Dosage on the Duration of Activity of Aqueous Suspensions. The effect of increasing the dosage of aqueous suspensions of estrogens of different particle size is shown in Fig. 1, 2 and 3. Several interesting points are demonstrated in these figures.

1) In most cases in Fig. 1, 2 and 3 it is seen that, with suspensions, many of the rats do not go into estrus until after 3-4 days especially at the lower dosage levels. This is unlike the uniform 36 to 48 hour period to reach estrus which is found after injection of oil solutions. This indicates that the solubility of the crystalline particles is such that in some rats a threshold concentration in the blood is not reached until 24-48 hours after injection, *i.e.*, about 36 hours before a positive estrus smear was found. This threshold value was never reached in the case of some rats especially those receiving only 50 I.U. or 100 I.U. of the crystals.

2) With particles of 100 mesh size (Fig. 3) the duration of estrus increases with increasing dosage up to a dosage of 400 or 500 I.U. Above these concentrations the duration of estrus was not lengthened appreciably by increasing the dosage. Unplotted data on an additional group of 20 rats receiving 2,000 I.U. showed results similar to those plotted for 400 I.U.

3) It can be seen in Fig. 3 that 50 I. U. (5 mcg) injected as a suspension of 100 mesh particles was more effective in most of the rats than 10,000 I. U. (1,000 mcg) injected in an oil solution. This indicates an increase of the order of 200 times in the efficiency of utilization of the estrogen in the case of the suspension relative to an oil solution.

4) With 200 mesh particles (Fig. 2) usually a shorter duration of estrus was found than with 100 mesh particles. A dosage of 200 I.U. of 200 mesh crystals was found to be as effective as 2,000 I.U. of the same but was superior to 100 I.U. and 50 I.U. A dosage of 100 I.U. of particles of 200 mesh size was found to be more effective than 10,000 I. U. in oil.

5) With the 325 mesh particles (Fig. 1) the

duration of effectiveness was considerably reduced from that found with larger particles and did not differ much with respect to dosage levels. Again, as little as 50-100 I.U. of the suspensions were found to be as effective as 10,000 I.U. in oil. However, 2,000 I.U. of the suspension were no more effective. This indicates the important role that particle size plays in controlling the duration of activity with these suspensions. Particle size was more important than the total dosage in determining the duration of action above a dosage level of 100 units.

Effect of Particle Size on Duration of Estrus. Data on the effectiveness of suspensions of different particle size at a selected dosage level upon the duration of estrus is presented graphically in Fig. 4. The greater duration of estrus as the particle size increases is obvious.

In Table I is shown some of the pertinent data relative to the particle size obtained with screens of the mesh size used. There is a better correlation between the duration of estrus and the linear size of the sieve opening than with the square or the cube of the sieve opening. Since estrone crystallizes as flat plates it is probable that the proportionality found is actually due to the smallest dimension of the crystals which is the thickness of the plates. The thickness of the particles could not be measured but it is probably proportional to the mesh size.

Note that single particles of 100 mesh size might weigh 1 to 3 μg and of 60 mesh size might weigh 10-15 μg . Hence only a few particles are required to make 100 I.U.

Effect of Particle Size on the Duration of Estrus in Immature Ovariectomized Rats. Estrus smears show only that sufficient estrogen is present to produce a positive vaginal smear and in no way indicate how much estrogen is present in excess as uterine weight would. Ovariectomized immature rats were therefore, given single injections of suspensions as indicated in Fig. 5 and were killed in groups of four. The uteri were excised and weighed. As with the estrus smears in the adult castrate rats it is apparent that 100 mesh particles have a longer duration of effect than 325 mesh particles. The duration of effectiveness of the 100 mesh particles is 14 days, a

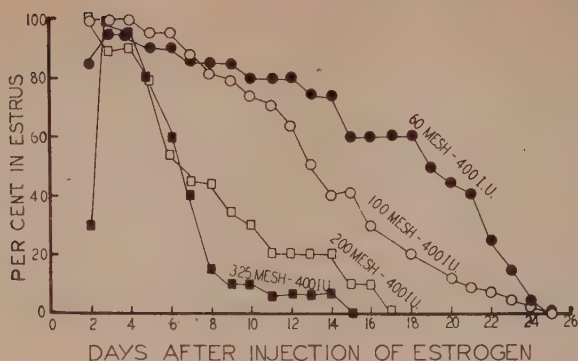


FIG. 4.

Duration of estrus in adult ovariectomized rats as a function of particle size at a dosage level of 400 I.U. (40 mcg). 40 rats were used in the 60 mesh-400 I.U. experiment. The other curves also appear in Fig. 1, 2 and 3.

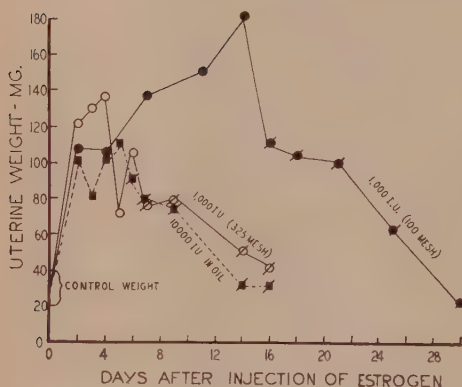


FIG. 5.

Uterine weight increase in immature ovariectomized rats as a function of time after a single injection of estrogen of different particle sizes. A bar through the point indicates all rats out of estrus. Each point represents average obtained on 4 rats.

period which agrees with the data obtained with estrus smears on adult ovariectomized

rats. The duration of effectiveness of the oil solution (10,000 I.U.) and the 325 mesh particles (1,000 I.U.) is approximately 5-6 days, a period which is also similar to that obtained in the adult ovariectomized rats. The superiority of the larger sized particles is again obvious and the increase of effectiveness of 10-100 times is also confirmed.

The uterine weight appeared to increase as long as an effective concentration of estrogen was present. There appeared to be a rapid decrease in uterine weight immediately after the estrogen stimulus ceased. Concomitant with this sudden drop in weight, negative estrus smears were obtained. Atrophy of the anestrus uterus then proceeded more slowly during a period of about 2 weeks back to a control weight level.

Summary. The duration of effectiveness of a single injection of aqueous suspensions of a crystalline ketonic estrogen preparation of 60, 100, 200 and 325 mesh particle size was

TABLE I.
Showing the Correlation Between Particle Size and Duration of Estrus.

Sieve No. (mesh/inch)	Sieve opening (μ)	Area of sieve opening (μ) ²	Max. wt of particles* (μ g)	Duration of estrus† (days)
60	250	62,500	15.6	19
100	149	22,208	3.3	14
200	74	5,476	0.4	8
325	44	1,936	0.08	5-6

* Wt calculated on basis of a cube of the dimensions of the sieve opening and a density of 1.

† Approximately the day on which 50% of the group of rats were out of estrus with 400 to 2,000 I.U.

compared with that of an oil solution of the same preparation. The dosages of the suspensions were varied from 50 I.U. to 2,000 I.U. Uterine weight or estrus smear were used as the criteria of effectiveness of the estrogens. 800 rats were used in the vaginal smear studies.

With the larger sized particles (60 and 100 mesh) 100 I.U. were as effective as 10,000 I.U. in oil solution. When the dosage level of 400 I.U. was used, estrus was maintained in 50% of the rats for 19 days with 60 mesh

particles, 14 days with 100 mesh particles, 8 days with 200 mesh particles and 5-6 days with 325 mesh particles. Further increase in dosage levels up to 2,000 I.U. did not increase the duration of the estrus. This indicates the primary importance of particle size with this estrogen in controlling the duration of its effectiveness.

We wish to acknowledge the cooperation of Dr. R. F. Varney of the Biological Control Department in these studies.

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Metabolism of Various 8-Aminoquinolines in Rhesus Monkey.* (17751)

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The following observations on metabolic transformations of various 8-aminoquinoline derivatives were an unexpected outgrowth of routine studies on the subacute toxicity of 2-methoxy-8-(3-aminopropylamino)-quinoline (CN 1100) for the rhesus monkey. These studies showed that, as compared with some 200 other 8-aminoquinolines which had been examined previously(1,2), CN 1100 had remarkably low toxicity, producing no untoward reactions when administered orally in repeated daily doses as large as 384 mg base per kg body weight.[†] Since the concentrations of this quinoline in the plasma were unusually low, even at doses as large as the above, and since less than 0.5% of the administered drug could be recovered from the urine, the question was raised whether the lack of toxicity of CN 1100 merely reflected poor absorption

from the gastrointestinal tract. This possibility led to a study of the toxicity of CN 1100 administered parenterally. It was found that the drug had little toxicity when given intramuscularly in single doses as large as 48 mg per kg, suggesting that the low toxicity observed previously was a real characteristic of CN 1100 rather than an artifact resulting from defective absorption.

As part of the above study on parenteral toxicity, measurements were made of the rate of disappearance of CN 1100 from the plasma. The 8-aminoquinoline concentrations were determined by the method of Brodie(3) which employs primary extraction of the quinoline with an organic solvent, followed by re-extraction and coupling of the drug with diazotized sulfanilic acid to yield a colored complex. In anticipation of the results to be obtained by this procedure, one of us (HBH) added diazotized sulfanilic acid directly to diluted samples of plasma and noted the development of colors which appeared far more intense than those obtained later in the analysis of the ethylene dichloride extracts. This impression was confirmed by quantitative studies which showed clearly that the plasma of monkeys receiving CN 1100 contained considerable quantities of an aqueous alkali-soluble, ethylene dichloride-insolu-

* This study was supported in part by grants-in-aid from the National Foundation for Infantile Paralysis and from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service.

1. Wiselogle, F. Y., *Survey of Antimalarial Drugs, 1941-45*. J. W. Edwards, Ann Arbor, Mich., 1946.

2. Unpublished observations, this laboratory.

† Limitations in the supply of CN 1100 precluded studies at higher doses.

ble degradation product (or products).

Current interests in the metabolic fates of certain 8-aminoquinolines made it important to determine whether the *in vivo* formation of alkali-soluble, ethylene dichloride-insoluble degradation products is a property peculiar to the 2-methoxy-8-aminoquinolines, of which CN 1100 is a representative, or whether the reaction is common to the 6-methoxy-8-aminoquinolines now employed as curative antimalarial drugs. Studies have been carried out with representative groups of both series of compounds. The results are outlined below.

Experimental. A. Metabolic transformations following intramuscular administration. Pamaquine [6-methoxy-8-(4-diethylamino-1-methylbutylamino)-quinoline], isopentaquine [6-methoxy-8-(4-isopropylamino-1-methylbutylamino)-quinoline], primaquine [6-methoxy-8-(4-amino-1-methylbutylamino)-quinoline], pentaquine [6-methoxy-8-(5-isopropylaminopentylamino)-quinoline], SN 13,058 [2-methoxy-8-(3-diethylaminopropylamino)-quinoline], and CN 1100 were administered in doses of 6 mg base per kg body weight to rhesus monkeys (*Macaca mulatta*), weighing 4.0 to 6.0 kg.[†] In addition CN 1100 was administered in doses of 12 and 48 mg per kg. Each of these compounds was employed as the soluble acid salt, pamaquine as the monocitrate, isopentaquine as the mono-oxalate, primaquine and pentaquine as the diphosphates, SN 13,058 as the dihydroiodide, and CN 1100 as the monophosphate. Aqueous solutions of these salts, containing 10 mg base equivalent per ml, were injected into the thigh muscles. Oxalated blood samples, drawn so as to prevent hemolysis, were obtained from the various monkeys prior to drug administration and $\frac{1}{4}$, $\frac{1}{2}$, 1, and 2 hours thereafter. Plasma samples were separated immediately and were analyzed for their contents of 8-aminoquinoline derivatives soluble in ethylene

dichloride by the Brodie method (3), modified by using ethylene dichloride in place of petroleum ether as the organic solvent.[§] The samples were also analyzed directly for their contents of total water-soluble 8-aminoquinoline derivatives which would couple with diazotized sulfanilic acid. The procedures used in the latter analyses were as follows. A 0.4 ml aliquot of plasma was added to 5 ml of diazotized sulfanilic acid reagent (3) and allowed to react at room temperature for the appropriate period to insure maximum color development. This color was measured in a Klett photoelectric colorimeter, using as blanks the pretreatment specimens of plasma which had been reacted with the diazotized sulfanilic acid reagent precisely as the post-treatment specimens. The equivalent 8-aminoquinoline concentrations of the plasma samples were calculated by comparing the above colorimetric readings with those obtained by analysis of plasma to which known concentrations of the respective drugs had been added. Studies carried out prior to the animal experiments showed that each of the compounds could be recovered quantitatively from plasma by either of the above methods.

The results of these experiments, summarized in Table I, show that each of the 8-aminoquinolines gave rise to alkali-soluble, ethylene dichloride-insoluble degradation products. On the 6 mg per kg doses, CN 1100 yielded the largest amount of degradation product, SN 13,058 the smallest amount.

The time relationships between the concentrations of substances soluble and insoluble in ethylene dichloride deserve comment. In all cases the peak concentrations of ethylene dichloride-soluble substances (unchanged drug?) were reached $\frac{1}{4}$ to $\frac{1}{2}$ hour after in-

3. Brodie, B. B., Udenfriend, S., and Taggart, J. V., *J. Biol. Chem.*, 1947, v168, 327.

[†] We are indebted to the following for the compounds used in this study: to Dr. Robert C. Elderfield, Columbia University, for pamaquine and CN 1100; to Dr. Nathan Drake, University of Maryland, for pentaquine; to Dr. J. B. Koepfli, California Institute of Technology, for SN 13,058; and the Lilly Research Laboratories, Eli Lilly and Company, for isopentaquine and primaquine.

[§] A 30-minute reaction period was required for full color development of the diazonium complexes of pamaquine, isopentaquine, primaquine, and pentaquine. The resulting yellow color was measured in a Klett colorimeter using a No. 47 filter. A 3-hour reaction period was required for full color development of the diazonium complexes of SN 13,058 and CN 1100. The pink color of these complexes was measured with a No. 54 filter.

TABLE I.
Concentrations of Various 8-Aminoquinolines and Their Metabolic Products in Plasma Following Intramuscular Administration.

Monkey No.	Drug	Dose, mg/kg	Mg of 8-aminoquinoline* per liter of plasma									
			A			B				C		
			Water-soluble substances			Ethylene dichloride-soluble substances				Alkali-soluble ethylene-dichloride-insoluble derivatives (A-B)		
			1/4	1/2	1	2	1/4	1/2	1	1/4	1/2	1
2912	Pamaquine	6	†	†	5.47	4.51	†	†	0.93	†	†	2.48
2925	Isopentaquine	6	2.94	4.70	5.29	5.88	2.82	3.10	1.67	0.12	1.60	2.19
2670	Primaquine	6	2.77	3.32	4.43	6.09	1.10	1.16	0.89	1.67	2.16	3.28
2926	Pentaquine	6	1.90	3.17	4.12	5.07	0.81	1.29	0.55	1.09	1.88	3.23
2828	SN 13,058	6	—	1.84	2.07	3.91	1.30	1.47	1.31	—	0.53	0.76
2657	CN 1100	6	7.07	14.14	16.02	5.82	1.64	1.56	0.92	5.43	12.58	15.10
2952	"	12	9.85	15.88	14.12	3.82	0.99	1.20	0.53	8.86	14.68	13.59
2607	"	48	23.82	63.97	87.32	53.83	2.61	3.54	2.97	21.21	60.43	84.35

* Expressed as parent drug equivalent.

† Animal in convulsions at this period; unable to obtain blood samples.

jection of the 8-aminoquinoline and declined substantially thereafter. In contrast to this, the concentrations of the alkali-soluble, ethylene dichloride-insoluble substances (degradation product or products) were lowest at the 1/4 hour period and, with the exception of CN 1100, rose steadily throughout the period of observation to reach an apparent peak at 2 hours. In the case of CN 1100, peak concentrations were attained at 1 hour.

It should be mentioned, also, that the peak concentrations of degradation products derived from any of the 8-aminoquinolines were always as great as the peak concentrations of unchanged drug and were considerably greater in some instances, notably with primaquine, pentaquine, and CN 1100. In the 2 monkeys, Nos. 2657 and 2607, receiving the latter compound, the peak concentrations of degradation product were 10 and 24 times the concentrations of unchanged drug. The absolute values of these concentrations were so great as to suggest that the degradation products may have greater chromogenic powers than the parent drug. The only other possible explanation for these high concentrations would be preferential localization of the drug in the plasma; this suggestion is not supported, however, by existing information on the tissue distribution of 8-aminoquinolines(4).

B. Metabolic transformations following oral administration. The above findings made it of interest to determine whether similar patterns of degradation occurred following oral administration of the various 8-aminoquinolines, also whether the degradation products accumulated in the plasma. In this experiment, pamaquine, isopentaquine, primaquine, pentaquine, SN 13,058, and CN 1100 in daily doses of 12 mg base per kg body weight were given to monkeys for 4 days. The daily doses were administered via stomach tube in equally divided portions at 8 A.M. and 5 P. M. Samples of oxalated blood were obtained on the final day just prior to the 8 A.M. treatment and 2 hours thereafter. Plasma samples therefrom were analyzed for their contents of water-soluble and ethylene dichloride-soluble 8-aminoquinoline derivatives, according to meth-

TABLE II.
Concentrations of Various 8-Aminoquinolines and Their Metabolic Products in Plasma Following Oral Administration.

Monkey No.	Drug*	Mg of 8-aminoquinoline† per liter of plasma					
		A		B		C	
		Water-soluble substances		Ethylene dichloride-soluble substances		Alkali-soluble, ethylene-dichloride-insoluble derivatives (A-B)	
		2	15	2	15	2	15
2912	Pamaquine	2.17	.17	.550	.103	1.62	.07
2922	Isopentaquine	4.28	.92	.292	< .017	3.99	.92
2738	Primaquine	9.07	2.59	1.737	.172	7.33	2.42
2924	Pentaquine	4.79	.50	.187	.031	4.60	.47
3012	SN 13,058	9.16	.24	3.565	< .012	5.60	.24
3025	CN 1100	3.46	< .11	< .010	< .010	3.46	.0

* In all cases the dose of drug was 6 mg per kg, administered at 8 A.M. and 5 P.M. daily for 4 days.

† Expressed as parent drug equivalent.

ods described above.

The results of this experiment, summarized in Table II, show that degradation products soluble in aqueous alkali but insoluble in ethylene dichloride were formed after oral administration of the various 8-aminoquinolines as well as after parenteral administration. The data also indicate that, with the possible exception of primaquine, there was no significant accumulation of these products in plasma.

C. Nature of metabolic products. Preliminary experiments, designed to elucidate the nature of the degradation products, have been carried out with drug CN 1100. Although these studies have not yet led to definition of the structures of the degradation products, they have shown that comparatively large quantities of alkali-soluble, ethylene dichloride-insoluble degradation products are eliminated in the urine of monkeys treated with CN 1100, also, that these degradation products may be extracted quantitatively from acidified urine with butanol.

The following experiment is characteristic of those which led to the above conclusions. Forty mg of CN 1100 base were administered to a monkey intramuscularly, after which urine was collected for a period of 18 hours. This urine contained 0.056 mg of ethylene dichloride-soluble 8-aminoquinoline derivatives (unchanged drug-?)—the equivalent of only 0.14% of the material administered. On the other hand, alkali-soluble, ethylene dichloride-insoluble substances (degradation

products) amounted to 21.07 mg—equivalent to 52.7% of the injected dose. Following acidification of the urine to pH 2, this product was extracted quantitatively with n-butanol. This solubility suggests that the material (or materials) is acidic in nature. Such a property could be acquired either by stepwise oxidation of the CN 1100 side chain to yield a carboxyl group or by cleavage of the quinoline nucleus to yield an anthranilic acid type structure. Work on these possibilities is in progress.

Discussion. The data set forth in the preceding experiments indicate clearly that certain 2-methoxy-8-aminoquinolines are degraded by the monkey to alkali-soluble, ethylene dichloride-insoluble derivatives. Observations with the 6-methoxy-8-aminoquinolines in current use as curative antimalarial drugs suggest, grossly at least, that the degradation of these compounds follows a similar pattern.

Although the nature of the degradation products has not yet been established, the present observations may be useful in pointing the way for future studies on the metabolism of the 8-aminoquinolines. Numerous groups of investigators, including our own, have pursued this general problem, believing that the elucidation of the metabolic fate of these compounds will materially assist the rational development of more effective and less toxic curative antimalarial drugs. Unfortunately, the possibility that the parent drugs are degraded to acidic substances has re-

ceived little attention from the laboratories engaged in these studies. The results of the present work focus attention on this particular group of degradation products.

Summary. Studies on the metabolism of the 8-aminoquinolines, pamaquine, isopentaquine, primaquine, pentaquine, SN 13,058, and CN 1100, have been carried out in the rhesus monkey. As shown by differential analyses of the 8-aminoquinoline contents of plasma, each of the above compounds is degraded to alkali-soluble, ethylene dichloride-insoluble de-

rivatives. This degradation follows either oral or intramuscular administration of the drugs. The extent of degradation of the different compounds varies markedly, being greatest with CN 1100 and least with SN 13,058. Preliminary studies on CN 1100 have indicated that the degradation products of this compound are present in the urine in considerable quantity.

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Distribution of Mercury in Rats Following Oral and Intravenous Administration of Mercuric Acetate and Phenylmercuric Acetate.* (17752)

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(Introduced by A. J. Lehman.)

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Chronic toxicity investigations in rats over a 2-year period have shown that mercury in the form of the phenylmercuric acetate salt is far more toxic than mercury in the form of the mercuric acetate salt(1). The following experiments were undertaken in an effort to trace the fate of the 2 mercurials following oral and intravenous introduction into the rat.

Method. Young adult male rats, ranging from 250 to 300 g body weight were used in these experiments. Equivalent quantities of mercury, in the form of aqueous solutions of phenylmercuric acetate and mercuric acetate were administered under very light ether anesthesia, either by the femoral vein or by stomach tube. In all but a few preliminary experiments, the dose of mercury was 120 μ g per animal. For the intravenous injection the dose was contained in 0.25 ml volume, for the oral, in 2.0 ml volume. The animals were sacrificed at intervals of 2, 6, 24, 48 and 96 hours after treatment; tissues, blood, and

excreta were analyzed for mercury. In the case of the excreta, urine and feces were collected separately, and the contents of the bladder and of the cecum and large intestine at the time of sacrifice were considered part of the respective voided samples. Mercury analyses were made by the method of Laug and Nelson(2).

Results. Table I shows the tissue distribution of mercury 24 hours after intravenous injection of the metal in the form of the 2 salts under investigation. It can be seen that the kidney is the most important storage depot for mercury and that the amounts found in this tissue are fairly well graded to the quantities of mercury injected. This storage response of the kidney after intravenous injection of mercury is, as might be expected, similar to that obtained when the metal is introduced by other routes, such as the dermal(3), vaginal(4), and oral(1).

* A part of these data was presented at the meetings of the Federation of American Societies for Experimental Biology, March, 1949.

1. Fitzhugh, O. G., Nelson, A. A., Laug, E. P., and Kunze, F. M., *J. Pharm. and Exp. Therap.*, 1950, in press.

2. Laug, E. P., and Nelson, K. W., *J. Assn. Off. Ag. Chem.*, 1942, v25, 399.

3. Laug, E. P., Vos, E. A., Umberger, E. J., and Kunze, F. M., *J. Pharm. and Exp. Therap.*, 1947, v89, 42.

4. Laug, E. P., and Kunze, F. M., *J. Pharm. and Exp. Therap.*, 1949, v95, 460.

TABLE I.
Distribution of Mercury in the Rat 24 Hours After Intravenous Injection of Mercury as
Mercuric Acetate and Phenylmercuric Acetate.

Dose, μg mercury	μg mercury per g of tissue						
	Kidney	Liver	Blood	Spleen	Muscle	Bone	Brain
Phenylmercuric acetate*							
120	13	.93	0	0	0	0	0
60	9.1	.52	.22	.66	0	.17	.51
30	5.4	.44	.16	0	0	0	0
10	2.2	.14	0	0	0	0	0
Mercuric acetate*							
120	19	.69	0	0	0	0	0
60	7.0	.17	.07	0	0	0	0
30	2.6	.10	.19	0	0	0	0
10	2.4	.06	0	0	0	0	0
Control*							
0	.30	.06	0	0	0	0	Tr

* Represents the average of 4 animals at each dosage level. Tr = Trace.

Reference to Table I also shows that other tissues, such as spleen, muscle, bone, and brain, are of relatively minor importance as storage depots for mercury. Fig. 1 shows that the storage of mercury in the kidney is markedly different for the 2 compounds within the first 24 hours. Thus, 2 hours after the intravenous injection of 120 μg of mercury as mercuric acetate, there is approximately

4 times as much mercury within this organ than after phenylmercuric acetate. With time, the mercuric acetate storage curve tends to fall, while the phenylmercuric acetate storage curve tends to rise, so that after 48 hours, they have converged to approximately the same value. With the exception of the 48 and 96-hour values, all storage differences between compounds are statistically signifi-

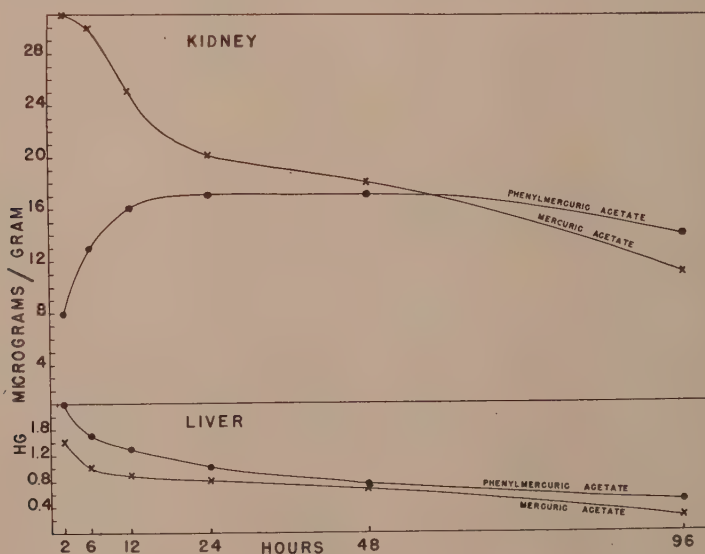


FIG. 1.

The storage response of the kidney and the liver of the rat to the intravenous injection of 120 μg of mercury in the form of mercuric acetate and phenylmercuric acetate. Each point represents average of 8 animals.

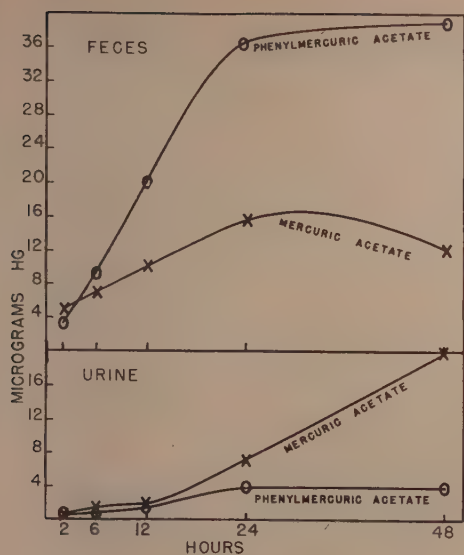


FIG. 2.

The excretion of mercury in the urine and feces following intravenous injection of 120 μg of mercury in the form of mercuric acetate and phenylmercuric acetate. Each point represents average of 3 animals.

cant. It is interesting to note that the storage response of the liver for the 2' compounds is reversed, that is to say, the initial storage of mercury after phenylmercuric acetate is significantly higher than after mercuric acetate. It should be noted that except for the first 24 hours, the subsequent loss in stored mercury from kidney and liver is relatively slow.

Fig. 2 describes the urinary and fecal excretion of mercury following intravenous injection of phenylmercuric acetate and mercuric acetate. It can be seen that urinary excretion of mercury is significantly greater after mercuric acetate than after phenylmercuric acetate, the differences being most marked in the second 24-hour period. In the feces, however, the situation is reversed, the largest amount of mercury being found after phenylmercuric acetate, with virtual completion of excretion within the first 24 hours.

Table II shows that the larger amounts of mercury in the feces after phenylmercuric acetate appear to originate in the small intestine, where it can be seen that the

mercury content after phenylmercuric acetate is significantly higher than after mercuric acetate. Secretion of mercury into the stomach does not appear to offer an explanation for this observation, for a number of analyses of gastric contents indicate a small mercuric concentration of about the same order for both compounds.

Table III shows the distribution of mercury in kidney, liver, blood, urine and feces at various periods following oral and intravenous administration of mercuric acetate and phenylmercuric acetate. With mercuric acetate, the difference between intravenous and oral administration is very well marked, especially in the mercury content of the kidney. In contrast, the differences between the amounts of mercury found after oral and intravenous injection are not nearly as marked with phenylmercuric acetate. These observations, together with the fact that only about half as much mercury is found in the feces within the first 24 hours after phenylmercuric acetate as that after mercuric acetate would indicate far greater absorption of the former salt. This is further pointed up by the difference in the blood levels after oral administration. In this connection one interesting observation may be made of the blood mercury levels 2, 6 and 12 hours after intravenous injection of either salt. It can be seen that while progressive changes in kidney and liver mercury are occurring during this period, the blood mercury remains practically fixed.

Discussion. Two salient facts may be noted from the above results. 1. Phenylmercuric acetate is much more readily absorbed from the intestinal tract than mercuric

TABLE II.

Total Mercury Content of the Small Intestine Following Intravenous Injection of 120 μg of Mercury as Mercuric Acetate and Phenylmercuric Acetate.

Hr after inj.	Mercuric acetate	Phenylmercuric acetate
	μg mercury in total contents	
2	5.5	9.4
6	4.7	12
12	4.5	8.1
24	2.4	3.7
48	2.0	2.4

Each value represents the average of 3 animals.

TABLE III.
Comparative Tissue Distribution of Mercury Following Oral and Intravenous Administration of 120 μg of Mercury as Mercuric Acetate and Phenylmercuric Acetate.

Hr		Mercuric acetate					Phenylmercuric acetate				
		Kidney, $\mu\text{g/g}$	Liver, $\mu\text{g/g}$	Blood, $\mu\text{g/g}$	Urine total, μg	Feces total, μg	Kidney, $\mu\text{g/g}$	Liver, $\mu\text{g/g}$	Blood, $\mu\text{g/g}$	Urine total, μg	Feces total, μg
2	(I) (O)	28 0.79	.95 .13	.60 .03	.39 .12	5.4	4.2 5.3	1.8 1.2	1.9 0.33	.43 .64	1.7 9.0
6	(I) (O)	28 1.5	.73 .13	.63 .14	1.3 .48	7.1 75	11 7.1	1.1 1.1	1.8 .52	.74 2.7	9.3 24
12	(I) (O)	26 1.5	.54 .16	.60 .09	1.8 .18	10 80	14 10	.85 .81	1.9 .46	1.4 2.3	19 37
24	(I) (O)	22 1.4	.56 .24		7.0 .15	16 82	16 7.9	.78 .74		3.9 2.8	37 45
48	(I) (O)	19 1.6	.53 .10		20 1.5	12 95	14 9.5	.48 .58		3.9 5.3	40 78

(I) Intravenous. (O) Oral. Each value represents avg of 3 animals.

acetate. This is reflected in greater storage of mercury in the liver and kidney after phenylmercuric acetate than after mercuric acetate, and confirmed also in the chronic feeding tests conducted in this laboratory(1); 2. There are important differences in the behavior of the two salts when they are injected intravenously. For example, 2 hours after mercuric acetate (Table III), the mercury concentration in the kidney rose to 28 μg per gram. This is equivalent to a total mercury content of 56 μg , based on an average weight of 2 grams for both kidneys. Thus within 2 hours the kidneys account for about half of the dose of mercuric acetate; in contrast, less than 1/10 of the dose after phenylmercuric acetate. It seems likely that part of the initial high concentration of mercury in the kidney after mercuric acetate is metal "in transit". This is confirmed by the subsequent rise in urinary excretion which eliminates about 17% of the dose in 48 hours; in contrast only 3% after phenylmercuric acetate. It would appear, therefore, that the urinary tract is the favored route for excre-

tion of mercuric acetate. For phenylmercuric acetate the evidence points to the liver as the favored route of excretion of mercury. Thus the initial concentration in this organ is higher after phenylmercuric acetate than after mercuric acetate, resulting, perhaps, in a higher concentration in the bile, which in turn would account for the elevated intestinal and subsequent fecal concentrations of mercury. Roughly, one third of the dose of phenylmercuric acetate is excreted in the feces in 48 hours, but only 10% of the mercuric acetate.

Summary. 1. Phenylmercuric acetate is more readily absorbed from the gastrointestinal tract than mercuric acetate.

2. The favored route for the excretion of mercury after phenylmercuric acetate is via the intestinal tract.

3. The favored route for the excretion of mercury after mercuric acetate is via the urine.

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Effect of Cytochrome C Therapy on Resistance to Decompression and Drug Depression. (17753)

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Proger and coworkers(1) have reported that cytochrome C is effective in alleviating tissue anoxia. In view of these reports a study was undertaken to determine whether parenterally administered cytochrome C would alter the critical pressure below which an animal could not survive. A considerable species difference in this critical pressure has been observed(2). The determination would, therefore, appear to be sensitive enough to reflect variations in the efficiency of tissue oxygen utilization. Since the action of depressant drugs has been thought to be due to a production of anoxia(3), it was also deemed of interest to ascertain whether cytochrome C treatment would exert an antidotal effect in animals intoxicated with drugs of this type.

Experimental. The decompression chamber used in this investigation was a vacuum desiccator communicating with a large ballast jar which was connected to a vacuum pump. The pressure in the chamber, which contained soda lime to absorb carbon dioxide and water, was measured with a Hg manometer. A needle-valve,* by means of which the rate of decompression could be regulated, was interposed between the chamber and ballast jar. All experiments were conducted at room temperature which remained essentially constant. Cytochrome C was prepared by the method of Keilin and Hartree(4) and dialyzed against distilled water. The final concentration, determined by a method described by Potter(5),

was 5 mg of cytochrome C per ml of solution. The preparation proved to be active in a reconstituted succinoxidase system(6). Black mice were used in all experiments except those involving sodium barbital, for which white mice were used. The mice were of either sex and approximately the same age, having an average weight of 25 g. The rate at which the mice were decompressed is shown in Table I.

Two series of mice were used in the decompression experiments. In the first series, the pressure at which the last extensor thrust of the hind legs occurred was taken as the critical pressure, since this endpoint was very close to death(2). The experimental mice were injected intraperitoneally with one ml of the dialyzed cytochrome C solution. The control mice were similarly injected with an equal volume of distilled water. Each mouse was injected one hour prior to decompression. In the second series, each mouse was made to serve as its own control. This was accomplished by using a slightly earlier endpoint in the course of decompression so that subsequent restoration to normal atmospheric pressure would revive the mouse almost immediately. The endpoint chosen, which proved to be very definite, was the first extensor thrust of the legs following the onset of gasping. In this way the critical pressure for each mouse was determined following intraperitoneal injection of one ml of distilled water and again, 24 hours later, following intraperitoneal injection of one ml of the dialyzed cytochrome C solution. Each mouse was injected 2 hours prior to decompression.

To investigate the possibility of antagonism

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2. Stullken, D. E., and Hiestand, W. A., *Proc. Soc. Exp. Biol. and Med.*, 1943, v54, 260.

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* We are indebted to Dr. A. L. Bennett, Department of Physiology and Pharmacology, for the construction of this needle valve.

4. Keilin, D., and Hartree, E. F., *Biochem. J.*, 1945, v39, 289.

5. Potter, V. R., in *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, edited by Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Burgess Publishing Co., Minneapolis, 1945, 191.

6. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.

TABLE I.
 Summary of Results Obtained with Cytochrome C.

A. Rate of decompression								
Time in min.:		0	3	6	9	12	15	18
Barom. press. mm Hg:		atm. press.	398	266	195	156	130	112
B. Effect of Cytochrome C therapy on critical barometric pressure								
Group		No. of mice	Avg pressure (mm Hg)			Range	S.D.	
First series	Control	7	177			160-198	14.7	
	Cyt. C treated	10	175			145-202	22.8	
Second series	Control	21	184			160-228	16.8	
	Cyt. C treated	21	183			140-225	18.6	
C. Effect of Cytochrome C therapy on time required for loss of righting reflex								
Group		No. of mice	Avg time (min.)			Range	S.D.	
20% alcohol (0.2 ml/10 g)	Control	10	2.2			0.9-6.2	1.5	
	Cyt. C treated	7	3.2			2.1-4.0	0.7	
Sodium pentothal (38 mg/kg)	Control	12	2.4			1.5-4.2	0.6	
	Cyt. C treated	13	2.9			0.8-5.5	1.6	
Sodium barbital (270 mg/kg)	Control	7	19			13-28	5.7	
	Cyt. C treated	6	20			16-23	3.8	

between cytochrome C and depressant drugs, 3 such drugs were employed: ethyl alcohol, sodium barbital, and sodium pentothal. The experimental mice were injected intraperitoneally with one ml of the dialyzed cytochrome C solution one hour prior to intraperitoneal injection of the drug. The control mice were similarly injected with an equal volume of distilled water. For each mouse the time interval following injection of the drug up to loss of the righting reflex was determined. The dosages employed are given in Table I.

Results and Comment. The cytochrome C treated mice at autopsy showed that the cytochrome C had been practically all absorbed from the peritoneal cavity. Moreover, when terminal failure of the bladder sphincter occurred during decompression, the urine was colored red. These observations indicate that the experimental mice were "saturated" with cytochrome C. It is apparent that the critical pressure observed with cytochrome C treated mice is the same as for control mice (Table I).

This is true even when each mouse serves as its own control. Furthermore, cytochrome C treatment had no effect on the response of mice to alcohol or to 2 types of barbiturates (short-acting and long-acting). Practically the same length of time was required for loss of the righting reflex in both experimental and control groups with each drug (Table I).

These observations do not support the hypothesis that cytochrome C therapy can increase the efficiency of tissue oxygen utilization and are in line with all other negative findings(7,8).

Summary. Cytochrome C did not alter the critical barometric pressure below which mice could not survive and was without effect on the depressant action of ethyl alcohol and of barbiturates.

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Electrokymographic Studies of the Relationship Between Electrical and Mechanical Events of the Cardiac Cycle.* (17754)

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(Introduced by Arthur C. DeGraff.)

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Studies of possible correlation between the electrical and mechanical events of the cardiac cycle have long been of interest. Wolferth and Margolies(1,2) and Nichol(3) measured the interval from the onset of the QRS to

the beginning of the carotid artery pulsation in the neck in cases of left bundle branch block and found this interval prolonged compared to the normal. From this they concluded that correlation did occur. Other investigators

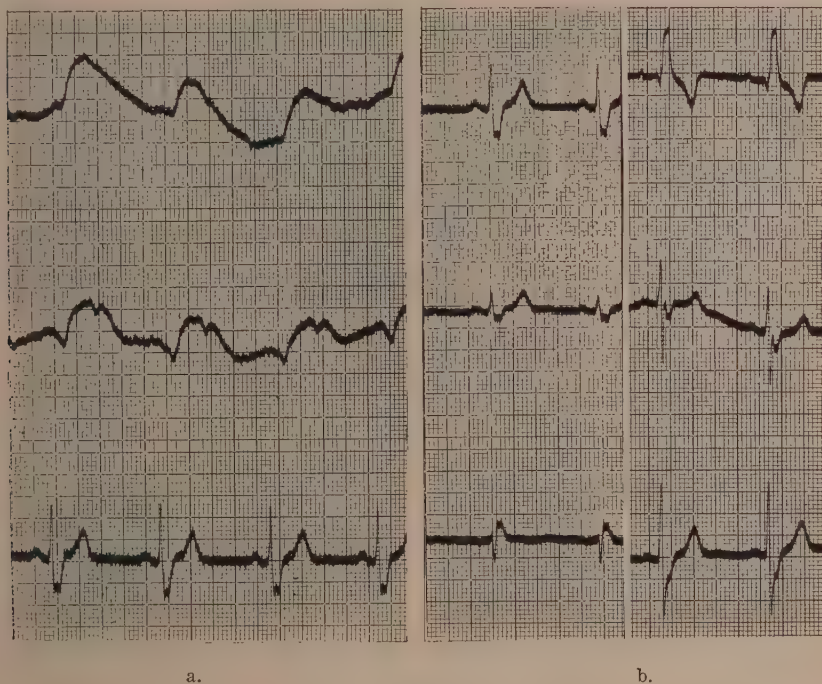


FIG. 1.

Case 11. Complete RBBB. a. The uppermost curve is an electrokymogram of the ascending aorta, the middle an electrokymogram of the pulmonary artery and the lowermost lead I of the electrocardiogram. The upstroke of the pulmonary artery is .01 second ahead of the upstroke of the ascending aorta. b. Electrocardiogram of case 11. The 3 standard leads are recorded simultaneously on the left side. On the right side from above down there is V1, V3, and V5, recorded simultaneously.

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the results of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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2. Wolferth, C. C., and Margolies, A., *Am. Heart J.*, 1935, v10, 425.
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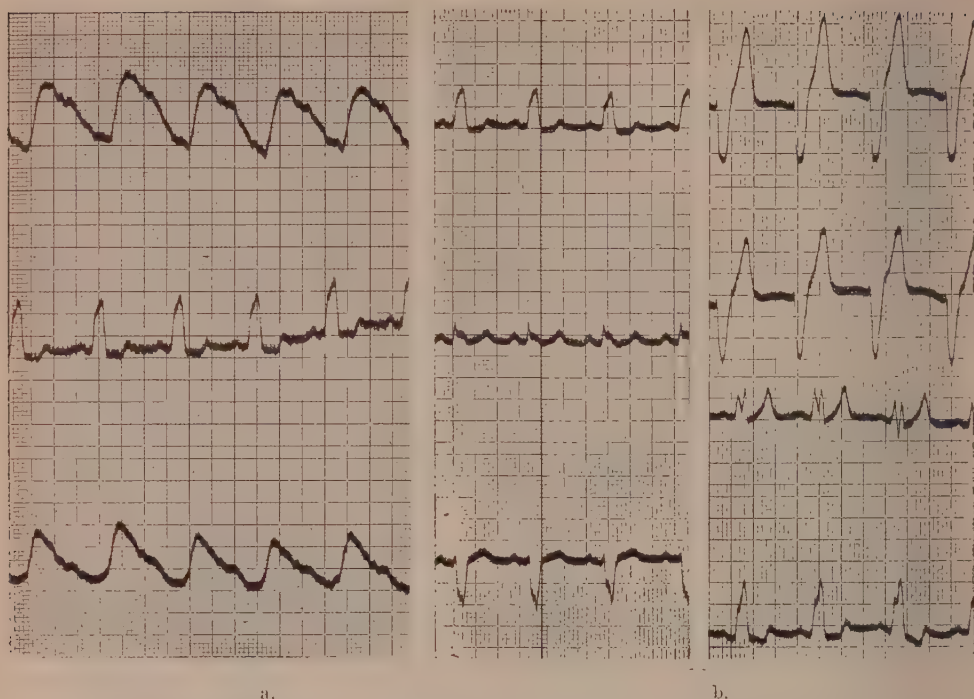


FIG. 2.

Case 23. Complete LBBB. a. The pulmonary artery upstroke (lowermost curve) is .02 second ahead of the upstroke of ascending aorta (uppermost curve). The middle curve is lead I. b. Electrocardiogram of case 23. The 3 standard leads are to the left; on the right from above down there is V1, V2, V5 and V6.

including Castex *et al.*(4), Kossman and Goldberg(5), Richards *et al.*(6), Ellinger *et al.*(7), and Coblenz *et al.*(8) have had similar experiences. On the other hand, Katz(9) and Battro(10) came to the opposite conclusions as a result of their studies.

With the development of the electrokymo-

gram(11-13), a new technic for study of this problem became available. We felt that re-investigation of this problem by the recording of electrokymograms of the pulmonary artery and ascending aorta simultaneously would be of great interest and value in deciding the issue. Simultaneous electrokymograms of the 2 great vessels allow an exact study of the onset of the ejection phase of the 2 ventricles without recourse to the carotid artery pulse as a reference point, thereby increasing the accuracy of the measurements. By means of a

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TABLE I.

Case No.	Results	Relations of pulmonary aorta and ascending aorta*	Correlation of electrical and mechanical events
A. Cases of incomplete RBBB, QRS duration			
1.	.11	+ .02	No
2.	.11	+ .04	"
3.	.11	— .06	Yes
4.	.12	+ .03	No
5.	.12	+ .05	"
6.	.12	— .02	"
B. Cases of complete RBBB, QRS duration			
7.	.13	— .04	Yes
8.	.13	+ .04	No
9.	.13	.00	"
10.	.14	— .09	Yes
11.	.14	+ .01	No
12.	.14	.00	"
13.	.15	.00	"
Cases of incomplete LBBB, QRS duration			
14.	.11	+ .05	Yes
15.	.12	+ .08	"
16.	.12	+ .03	No
17.	.12	+ .02	"
D. Cases of complete LBBB			
18.	.13	— .03	No
19.	.13	+ .06	Yes
20.	.14	+ .01	No
21.	.14	+ .01	"
22.	.14	+ .05	Yes
23.	.14	+ .02	No
24.	.15	+ .08	Yes
25.	.15	+ .02	No

* + means that the onset of the pulmonary artery upstroke precedes that of the ascending aorta.

— means the reverse.

new 3 channel direct-writing recording apparatus (the Technicon†), we have recorded the electrocardiogram simultaneously with the two elektokymograms. The tracings were taken with the patient sitting, and in the right anterior oblique position. In this position, on the anterior border of the heart, the ascending aorta and (below the ascending aorta) the pulmonary artery are readily visible. The photo-electric cells of the elektokymograph can easily be placed over the edges of the ascending aorta and pulmonary artery in this position, so as to obtain the proper tracings.

Results. It was, of course, first necessary to establish the normal relationship between the elektokymograms of the ascending aorta and pulmonary artery by this technic. In a study of more than 50 normals, we found that the normal range was from +.03 to —.02 second.

† We are indebted to the Technicon Corp., 215 E. 149th St., New York City, for the use of this instrument.

That is, pulmonary artery ejection might precede aortic ejection (as measured by the upstroke of the elektokymographic curves in both instances) by as much as .03 second, or might follow it by as much as .02 second. In 25 cases of bundle branch block, we found correlation between the electrical and mechanical events in only 8. In 17 cases the relation between the onset of the pulmonary artery upstroke and that of the ascending aorta was with the normal range listed above. In all cases the diagnosis of bundle branch block was made from the standard and at least 6 precordial leads, using the intrinsicoid deflection of the precordial leads to decide the side of bundle branch block. Mention must also be made of the fact that repetition of the curves at different times leads to the same results within the error of measurement of .01 second—the results are reproducible. Fig. 1, 2 illustrate some of our curves. In these figures, the distance between the thin vertical lines represents .04 second, and the

distance between the heavy vertical lines 0.2 second as on ordinary electrocardiograms. Our overall results are shown in Table I.

Attention should be drawn to Case 5. Here the QRS is .12 second and incomplete right bundle branch block is present. But the onset of the pulmonary artery upstroke is .05 second ahead of the onset of the upstroke of the ascending aorta, a result well outside the normal range, but in a direction opposite to that expected. In Case 18, with left bundle branch block (QRS .13), the onset of the upstroke of the ascending aorta is .03 second ahead of the onset of the upstroke of the pulmonary artery, again a relation outside the range of normal, but in a direction opposite to that expected.

We are continuing investigations of this problem in cases of ventricular premature beats and Wolff-Parkinson-White syndrome, but our results to date indicate lack of correlation between electrical and mechanical events in these instances also.

Summary. In a study of 25 cases of bundle branch block, we have found no correlation between the electrical and mechanical phases of the cardiac cycle in 17 cases. Furthermore, in two cases of bundle branch block, an abnormal mechanical relationship between the onset of ejection in the pulmonary artery and ascending aorta was noted in a direction opposite to that expected.

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Content and Distribution of Glycogen in *Schistosoma mansonii*.^{*} (17755)

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Carbohydrate metabolism of *Schistosoma mansonii* proceeds at an exceedingly high rate. These trematodes utilize in one hour an amount of glucose equivalent to almost one-fifth of their dry weight(1). In the course of investigations concerned with the carbohydrate metabolism of schistosomes, a study was made of the content and distribution of glycogen in these helminths.

Methods. The flukes were removed from the mesenteric and portal veins of mice infected 6 to 23 weeks previously by the intraperitoneal injection of 160 cercariae of *S. mansonii*. The worms were placed in an ultrafiltrate of ox serum. After separation of the males from the females, both sexes were divided into two groups. A predetermined number of one group (5 males or

30 females) was used for the determination of glycogen, another for that of the dry weight. Glycogen was determined by a previously described modification(2) of the method of Good, Kramer and Somogyi(3), or by the colorimetric procedure of Seifter and Dayton(4). In the latter procedure, filter No. 60 of the Klett-Sumerson colorimeter was substituted for filter No. 62, since this resulted in a higher sensitivity and in a better proportionality between extinction and glycogen concentration. Provided the glycogen was precipitated prior to colorimetric analysis, results obtained by the two methods agreed within 3%. The other group of worms was rinsed quickly in distilled water, placed in small tared cups and dried to constant weight at 90°C. Glycogen content was calculated from the total amount of glycogen found and the dry weight of a similar number

^{*} This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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of worms.

A histological study was made of the distribution and amount of glycogen in the various organs and tissues of adult schistosomes. The worms were fixed immediately in a freshly prepared solution consisting of saturated alcoholic picric acid (33 ml), concentrated formalin (6 ml), and glacial acetic acid (2 ml). Fixation time was about 18 hours. Dehydration was accomplished by 3 changes of absolute isopropyl alcohol followed by 2 changes of thirty minutes each in xylol. The tissues were first overstained in alum hematoxylin without bluing, dipped in Best's differentiating solution (methyl alcohol, 10 ml, and 50% ethyl alcohol, 45 ml), and then placed for about 15 minutes in the glycogen staining solution[†] until the granules were a vivid red. They were then differentiated by brief but thorough agitation, dehydrated in 2 changes of absolute isopropyl alcohol, cleared in xylol and mounted.

Results. The analytical data are recorded in Table I. Glycogen content of the males was 3 to 6 times greater than that of the females. In the latter the quantity of glycogen did not vary with the age of the worms. In contrast, the glycogen stores of the males tended to increase with age, although not in every instance.

The histologic distribution of glycogen was similar to that noted by Axmann(5), and the material occurred mainly in the form of fine or coarse granules. In all worms the deposit was limited principally to musculature, parenchyma, tubercles, and suckers. None was present in cuticle or subcuticle, excretory

[†] This is prepared from a stock solution consisting of carmine, 0.4 g; potassium carbonate, 0.2 g; potassium chloride, 1.0 g, and distilled water, 12 ml. The ingredients are mixed and brought to a boil. The solution is not removed from the flame until it has turned a deep red color and has developed a froth. After slight cooling, 4 ml of concentrated ammonium hydroxide are added. For use the above quantity of a stock solution is diluted with 15 cc of concentrated ammonium hydroxide and 15 ml of methyl alcohol. The mixture should become turbid on addition of the methyl alcohol.

5. Axmann, M. C., *J. Morphol.*, 1947, v80, 321.

TABLE I.
Glycogen Content of Schistosomes.

Weeks after inj. of cercariae	% glycogen (dry matter)	
	Males	Females
6	14.3	4.3
	15.8	5.0
	13.6	
	14.8	
7	15.6	4.2
	17.2	3.8
	19.6	4.6
	16.6	
8	18.7	4.5
	18.3	
	17.3	
9	18.7	4.8
	16.8	2.7
	17.8	4.3
	18.2	
	16.2	
10	17.6	
	16.1	4.3
	18.4	3.9
	18.8	4.6
11	17.3	
	18.4	4.8
	18.4	4.1
	19.3	
13	18.6	
	22.2	4.5
	18.4	4.2
	19.8	
	17.8	
14	20.2	
	18.7	3.9
	15.9	4.6
	15.8	4.3
	20.3	
16	19.6	
	18.8	
	17.7	4.4
	20.8	4.9
19	22.2	
	19.3	
	21.0	3.8
	17.1	4.5
	20.1	4.1
	22.2	4.3
	26.6	
22	22.4	
	22.6	
	21.4	
	23.2	
	22.6	4.2
	29.0	3.6
23	18.6	
	21.8	
	22.4	
	25.8	4.7
	21.5	4.0
	22.0	
	20.1	
	22.2	

system or sexual apparatus, although occasionally, there was slight interstitial deposit in the testis. The intestinal crura were essentially negative except for fairly frequent scant punctate granular deposits in the outer rim of cytoplasm of the lining cells.

Male and female worms showed a similar distribution of glycogen. However, the amount was distinctly less in the female than in the male and sometimes apparently out of proportion to difference in size. This is explained by the less well-developed musculature and parenchyma of the female worm and also by the relatively more abundant sexual apparatus which is glycogen negative.

The histological studies permitted only a rough quantitative estimate of the amount of glycogen present. In spite of this, the quantity of glycogen in male worms from the longer periods of infestation usually exceeded that present in the early stages. In the

former, the glycogen was often found in coarser, more compact granular form within musculature and body parenchyma. However, the location of the glycogen remained the same.

Summary. From 13.6 to 29.0% of the dry matter of the males of *S. mansoni* consisted of glycogen. In females the amount varied between 2.7 and 5.0%.

The glycogen in both male and female worms was deposited principally in the musculature and parenchyma. The relatively small amount in the female is explained by the less well-developed musculature and parenchyma and the more abundant sexual apparatus which is glycogen negative.

Histological studies were in general accord with the quantitative observation that the amount of glycogen in the male tends to increase with age.

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Studies of Influenza A (PR8) Infected Tissue Cultures by Electron Microscopy.* (17756)

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The growth and development of a virus may be studied in the cells upon which it is parasitic. In the course of such studies, by means of a tissue culture technic(1,2), we have found that there is a very close association between the formation of the typical spheres of influenza virus and the elongated rod-like filaments previously shown in centrifuged influenza infected allantoic fluid(3-6).

Previous observers have noted that rod-like forms occur in ultracentrifuged allantoic fluids infected with several strains of influenza virus including newly isolated strains (4,7,8) and that they adsorb and elute from red cells along with the spherical virus particles(7). Moseley and Wyckoff(4) have noted the occurrence of segmentation on the rods and consider this as evidence for a conversion between rod-like and spherical forms.

We have confirmed these observations and have noted rod forms in all three major types (Influenza A PR8, Weiss,[‡] W. S.,[‡] and FM

* Supported in part by a grant in aid from the National Institutes of Health, U.S.P.H.S.

[†] National Institutes of Health Fellow.

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FIGS. 1 and 2. Electron micrographs from areas of Influenza A. (PR8) infected tissue cultures where cell destruction was observed. Spherical and filamentous forms are seen in close association. Mag. $\times 12,900$. (Fig. 2 is unshadowed).



FIG. 3 (left). Electron micrograph of an area from an infected tissue culture with segmentation of filaments showing varying periodicities. ($\times 23,000$).

FIG. 4 (right). Another area showing suggestive evidence of a breakdown of the filaments and a release of spherical particles. ($\times 23,000$).

No. 1,[†] Influenza B Lee,[‡] and Swine Influenza V15) but not in allantoic fluid from normal eggs or those infected with Mumps, Newcastle disease virus, or Eastern Equine Encephalitis virus. We have extended this study to observations made directly on cells infected with influenza A (PR8) virus.

Method. Maximow flying cover slip cultures were used exclusively in this study following the technic of Porter, Claude and Fullam(1). Originally, plasma clot cultures were used, introducing the virus at the time the cultures were started, but after 10 experiments, averaging 4 infected and 4 control cultures each, we had seen only very occasional evidence of virus. At this point, in order to obtain a very high cell infection rate and to reduce the time in tissue culture to a minimum, the following method was adopted.

Seven or 8 day old chick embryos were inoculated onto the chorio-allantoic membrane with 0.2 cc of a suspension of influenza A (PR8) virus-containing 10^8 infective units per cc (10% allantoic fluid). This strain had

been originally obtained from Dr. R. E. Shope in 1946 and maintained by about 15 embryo passages since then. The eggs were incubated at 35°C for 24 or 48 hours and the chorio-allantoic membrane was removed, cut into small pieces, and set up in Maximow cultures using Simms' ultra-filtrate medium(9). The cultures were incubated at 35°C for 24 hours and fixed with 2% osmium tetroxide vapor for from 2 to 7 hours, washed, dried and shadowed with chromium. Five experiments have been done totaling 9 cultures from influenza infected eggs and controlled with 6 active and 6 inactive preparations of a pox virus, probably herpes simplex, and 6 active and 4 inactive preparations of Newcastle Disease (CG) virus. Inactivation was performed by heating to 65°C for 30 minutes.

Results. Fig. 1 and 2 show masses of the elongated forms mentioned above and seen in most of our tissue cultures in areas in which the cells were destroyed. The co-existence of typical spherical influenza particles and filaments is seen. Isolated instances of clumps

[†] Courtesy of Dr. T. G. Ward, Johns Hopkins University School of Hygiene.

9. Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **733**, 619.

of almost pure rods have been seen and one wandering cell, presumably a macrophage, showed a large preponderance of spherical forms, but the vast majority of infected cells show the co-existence of the two shapes.

In some favorable areas it was possible to see gradations between filaments and spheres. Fig. 3 shows rods with segmentation varying in period, the largest being approximately 100 $m\mu$ or the same as the spherical particles. Some seemingly "degenerate" filaments are shown in Fig. 4 with spheres spaced along them which suggest that segmented filaments may break down releasing spherical forms.

It is important to note that the number of segmented or transitional forms seen is much higher in these tissue cultures than in influenza virus preparations from allantoic fluid. Neither spherical nor rod-like forms have been seen in the controls.

Discussion. There is good evidence that the elongated filaments seen in influenza virus infections of the chick embryo are intimately connected with the development of the virus for 4 reasons. First, the rods have been seen in 6 old strains of influenza virus and in several newly isolated ones(7,8), all strains examined having shown them. Normal allantoic fluid from embryos infected with other viruses have not shown them; second, other

investigators(7) have shown that the filaments adsorb and elute from red cells in the same way that the spherical virus particles do, an observation which we have confirmed; third, we have shown in this article that on direct examination of tissue culture epithelial cells bearing the infection, filaments and spheres appear in close association with each other; fourth, segmented or beaded forms in various stages suggesting a transition from filaments to spheres have been demonstrated.

Summary. 1. Epithelium from influenza A infected chick chorio-allantoic membranes were grown in tissue culture and examined with the electron microscope.

2. The infected cells showed spherical virus particles in close association with elongated rod-like structures like those seen in ultra-centrifuged allantoic fluid from influenza infected eggs.

3. Various gradations between unsegmented rods and beaded chains were seen in certain areas strongly suggesting a link between the two forms.

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Thyroid Hormone and Tissue Cholesterol Distribution.* (17757)

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A number of investigators have demonstrated, both in humans and in experimental animals, that there is an inverse relationship between the activity of the thyroid gland and

the plasma cholesterol level(1). The influence of the thyroid hormone on the cholesterol concentration of other tissues, however, has been studied only to a very limited extent(2). In view of the importance of this problem for

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2. (a) Forbes, J. C., *Endocrinol.*, 1944, v35, 126; (b) May, L. G., Moseley, R. W., and Forbes, J. C., *Endocrinol.*, 1946, v38, 147; (c) Fleischmann, W., and Shumacker, H. B., *Bull. Johns Hopkins Hosp.*, 1942, v71, 175.

TABLE I.
Experimental Groups.

Group	Dietary supplements		Feeding period, wk	No. of rats
	Endocrine component	Cholesterol and Bile Salt		
I	—	—	—	17
II	—	+	26-38	9
III	Thyroid	—	6	5
IV	"	+	6-8	11
V	Thiouracil	—	30	7
VI	"	+	32	8

the etiology of arteriosclerosis, the effects of experimental hyper- and hypothyroidism on the cholesterol distribution in blood, liver, and aorta were investigated in rats fed a high cholesterol diet. The results are summarized in the present report.

Methods. Young adult rats (University of Southern California strain) were fed this laboratory's stock diet, slightly modified to contain more vitamin B complex(3). The experimental diets were supplemented either with desiccated thyroid, or with 0.25% thiouracil, each either alone, or in combination with 1% cholesterol and 0.5% bile salt. The hyperthyroid groups include animals treated with two different thyroid preparations; the first series received desiccated hog thyroid, containing 0.65% total I and 0.31% thyroxine, at a level of 0.4% of the diet; the food of the second group was supplemented with 0.6% of thyroid powder U.S.P. (Armour). The cholesterol was added to the diet dissolved in warm cottonseed oil, as reported previously(3). The experimental conditions are summarized in Table I. For purpose of comparison, data for control rats, and for rats fed cholesterol and bile salt for 26-38 weeks, are included (Groups I and II). These data were taken from a recent publication(4). Following feeding periods of 6-8 weeks for the animals fed thyroid (Groups III and IV), and of 30-32 weeks for the groups treated with thiouracil (Groups V and VI), the animals were autopsied (nembutal anaesthesia). Blood and liver were taken

for chemical determinations of total cholesterol, and the aorta for histological examination. The techniques employed for cholesterol extraction and determination, and for histological examination of the aorta were recently described(4). Löwenthal(5) stressed the importance of making histological studies for cholesterol as soon as the tissues are fixed, to avoid loss during storage in formalin. Our own observations confirm this point. Unfortunately, it was not possible, in the present work, to process in time all the aortas of the thyroid-fed rats (Groups III and IV); therefore, only the structural findings will be reported when examination was delayed.

Results. The experimental results are summarized in Table II. In all groups, the aorta appeared macroscopically normal. The doses of thyroid given were toxic and produced the expected symptoms, such as loss in weight, nervousness, rapid breathing, shivering and a shaggy coat. The mortality was high in the groups fed thyroid alone (III); in the first series of Group III, in particular, all rats died within a period of 5 weeks.[†] In the animals treated with the same doses of thyroid, but in addition with cholesterol and bile salt (Group IV), the symptoms appeared to be somewhat milder, and the mortality was significantly lower(3).

Thyroid hormone alone (Group III) caused a slight decrease in plasma cholesterol, but it had no significant effect on the sterol content of the liver. In the animals fed thyroid

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[†]Rats which died during the experimental period are not included in the data given in Table II.

TABLE II.
Experimental Results.

Group	Cholesterol content			Histologic changes of aorta No. of rats		
	Plasma, mg/100 ml	Liver, %	Liver wt, % of body wt	Showing microscopic changes		
				Examined	Cholesterol deposits	Intimal lesions
I	60 ± 3	.21 ± .02	3.26 ± .12	11	0	0
II	138 ± 12	2.03 ± .35	4.03 ± .19	9	2	2
III	34 ± 3	.27 ± .01	4.28 ± .30	4*	—	0
IV	45 ± 4	1.69†	4.96 ± .12	11‡	2	3
V	118 ± 18	.25 ± .01	3.92 ± .18	7	0	2
VI	251 ± 46	1.46 ± .25	5.97 ± .20	8	1	1

* Examined only on paraffin sections.

† Samples pooled.

‡ Four of these 11 specimens examined only on paraffin sections.

in combination with cholesterol and bile salt (Group IV), the plasma cholesterol was slightly higher than in Group III, yet still somewhat below normal. In spite of this low plasma level, a significant accumulation of the sterol occurred in the liver comparable in extent to the liver cholesterol content of the rats given cholesterol and bile salt without the addition of thyroid (Group II).

Microscopic examination of the aortas revealed that in group IV‡ five of seven rats had fine cholesterol needles scattered through the aorta, mostly in the intimal coat; in 2 of these animals the crystals were especially numerous. The aortas of the 2 remaining rats were merely loaded heavily with stainable fat.

The endothelium was thickened in 2 of 4 rats of Group III, and in all specimens of Group IV; in 3 of 11 animals of the latter group it was considered atheromatous.

Thiouracil alone (Group V) caused a significant increase in the cholesterol content of the plasma, without modifying the hepatic concentration. By far the highest plasma cholesterol values were observed in the animals fed both thiouracil and cholesterol and bile salt (Group VI). It is interesting, however, that in this group the cholesterol accumulation in the liver was not higher but, if anything slightly lower than in Group IV, in spite of an appreciably longer feeding

period.

Upon microscopic examination of the aortas in Group V, only one rat, a female with large fibroadenoma of the mamma, was found to have traces of cholesterol. The other animals of this group showed only moderate to large amounts of stainable fat in the intimal coat, and traces in the media.

In Group VI, localized birefringence was seen in the endothelium of 4 rats, marked only in one of these animals. In 3 of these rats, the media also contained birefringent crystals.

Spots of intimal thickening were observed in 2 of 7 specimens of Group V, and in one rat of Group VI. In the latter the hypertrophy was pronounced enough to be called a fatty plaque; underneath the plaque, the media carried stainable fat, and films covered the elastic lamellae. Two other rats of this group showed only minor intimal thickening. More severe vascular changes than those seen in the aorta were found in sections of lung tissue of Group VI.

The liver weight was increased as a consequence of cholesterol as well as of thyroid supplementation. A combination of the two agents produced an approximately additive effect (Table II, IV). Thiouracil alone caused only a moderate increase in liver weight; by far the largest livers were observed in the group fed both thiouracil and cholesterol and bile salt (VI).

‡ The specimens of group III were examined on paraffin sections only.

Discussion. The experimental results pre-

sented here indicate that the plasma cholesterol level depends on the concentration of circulating thyroid hormone, in agreement with earlier reports(1). The cholesterol content of the diet, on the other hand, does not seem to influence plasma cholesterol significantly in the hyperthyroid rat. The liver cholesterol content was practically normal in all groups fed the low cholesterol diet, and it was strikingly increased by the high cholesterol intake. In both cases, the liver sterol values were practically independent of the concentration of circulating thyroid hormone. These observations confirm results obtained by May and others on animals treated with thiouracil(2b). Forbes, however, reported that thyroxine tended to decrease the cholesterol content of the liver, as long as the choline intake was adequate(2a). In connection with the unexpected cholesterol distribution found in the hyperthyroid rats fed the high cholesterol diet, it is interesting that Fleischman and Shumacker reported that treatment with thyroxine of rats fed a low cholesterol diet caused a decrease of serum cholesterol, but an increase in the sterol content of the entire carcass(2c).

The finding that no gross changes were produced in the aorta, even in the cholesterol-fed hypothyroid rats—the animals showing the highest plasma cholesterol levels—is in agreement with a report by Horlick and Havel(6). These results confirm again that rats are highly resistant to cholesterol atheromatosis. It might be mentioned, in this connection, that in another resistant species, *i.e.*, the dog, Steiner and Kendall recently obtained grossly visible atheromata as a consequence of cholesterol and thiouracil feeding(7). A quantitative comparison between the results of the dog and rat experiments, however, is not possible because the experimental conditions were different. Microscopic cholesterol deposition in the aorta was seen in a few experimental animals. This deposition occurred in two forms, indicating

two types of structural alteration of the aortic wall. Hyperthyroid rats showed widely scattered cholesterol crystals, while thiouracil-fed animals possessed focal cholesterol accumulations. The appearance of cholesterol in the aortas of the hyperthyroid rats was unexpected, but it is in agreement with findings of Bruger and Fitz who performed experiments along similar lines, treating rabbits with pituitary thyrotrophic hormone(8). Other investigators, however, reported that thyroid hormone prevented or counteracted aortic cholesterol deposition and atheroma formation in rabbits and chickens fed a high cholesterol diet(9).

A comparison of the tissue cholesterol distributions shows that neither plasma and liver cholesterol concentrations, nor plasma cholesterol level and aortic cholesterol deposition ran parallel in the different experimental groups. On the other hand, hepatic and aortic cholesterol depositions followed a similar pattern.

Summary. The effects of high and low concentrations of circulating thyroid hormone on the total cholesterol contents of plasma and liver, and on aortic cholesterol deposition were studied in rats fed a diet either low or high in cholesterol.

In the hyperthyroid groups, the plasma cholesterol values were normal or slightly lower, and to some extent independent of the cholesterol content of the diet; in the thiouracil-treated series, the plasma cholesterol levels were significantly increased, reaching the highest values in the rats fed the high cholesterol diet.

The liver cholesterol concentrations were normal in the animals not supplemented with dietary cholesterol, and strikingly increased in all groups fed the high cholesterol diet, to a great extent independently of the concentrations of circulating thyroid hormone.

The aorta appeared macroscopically normal

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7. Steiner, A., and Kendall, F. E., *Arch. Path.*, 1946, v42, 433.

in all groups; microscopic examination revealed, in the cholesterol-fed animals, a moderate cholesterol deposition, diffuse in the hyperthyroid rats, and focal in the thiouracil fed animals. Isolated cases of early

atheromata and widespread fatty infiltration were observed in the rats fed thyroid and cholesterol, and in both groups treated with thiouracil.

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A Skin Test for Infectious Hepatitis.* (17758)

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Evidence has been obtained that the virus of infectious hepatitis (IH) can be propagated in minced chick embryo tissue culture and in the embryonated hen's egg(1,2). Virus thus cultivated induced mild hepatitis without jaundice in human volunteers but no laboratory test for the presence of virus has evolved as yet. However, as will be shown in this paper, amniotic fluid of chick embryos infected with IH virus may serve as skin test antigen with apparently specific results.

The Akiba strain of IH virus was passed 10 times through chick embryo tissue culture and then transferred in 0.2 ml amounts to the amniotic cavity of 8 day old chick embryos. After incubation at 37° for 7 days the whole contents of the eggs minus the albumen were emulsified in an approximately equal volume of saline by means of a Waring blender and passed amniotically to fresh embryos. After 3 such passages, when slight liver damage became apparent in some of the embryos, only amniotic fluids were harvested and passed in further transfers and the incubation period was reduced to 5 days. Amniotic fluids of the 6th and 7th consecutive passage were used for IH skin test antigens. Control materials

were prepared under identical conditions except for the fact that the amniotic series was initiated with buffered saline instead of the Akiba tissue culture material. The specific and control antigens were irradiated with ultraviolet light for a period 5 times that required to inactivate influenza virus. The materials were cultured aerobically and anaerobically, and tested for safety by intraperitoneal and intracerebral inoculation of mice and by intradermal injection of human volunteers with no history of IH. The IH and control antigens were stored at 4°C until used. They were administered in 0.1 ml amounts on the right and left forearm, respectively.

Results of 161 skin tests are shown in the Table. They were performed (a) in human volunteers in corrective institution No. 1 who, 3 months to 2 years previously had submitted to infection with IH virus as contained in stools and serum taken during the acute stage of IH (Akiba or NL Strain) or in tissue culture; (b) on volunteers in institution No. 2 injected 5 months to 5 years previously with the virus of serum hepatitis (SH); (c) on 62 random subjects of institution No. 1; (d) on 9 young children 2½ to 4 years old; and, finally, (e) on 10 cases of spontaneous IH 12 days to 6 months after onset of illness. Positive skin tests to IH were characterized by the development within 24 hours of an area of erythema of a somewhat brownish red tinge and of induration which as a rule exceeded 10 mm in diameter (4 to 45 mm) and which persisted, although decreasing in intensity, some-

* This investigation was conducted under the Commission on Liver Diseases, Armed Forces Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, U.S. Army.

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TABLE I.
Result of IH Skin Tests in Individuals with Past Histories of IH or SH and in Random Groups.

History of group	No. in group	No. showing negative control test	IH test	
			No. positive	% positive
Exposed to IH (I.* No. 1) Signs of illness	25	24	24	100
Exposed to IH (I. No. 1) No signs of illness	9	8	7	87.5
Exposed to SH (I. No. 2) Signs of illness	16	13	3	23
Exposed to SH (I. No. 2) No signs of illness	32	31	13	42
No exposure (I. No. 1)	62	56	23	41
No exposure (children)	9	9	1	11
Natural IH	10	10	10	100

* I = Institution.

times up to a period of one week. The control antigen caused no reactions in the skin in 149 of the cases reported here. However, in 12 of the individuals there were areas of pink erythema with little or no induration, measuring from 2 to 30 mm in diameter in 24 hours. These had, however, faded by the 2nd day in the majority of cases. The specific reactions in most instances appeared clearly separable from the non-specific ones by a difference in coloration, by the absence of marked induration and, frequently, by the size of the areas involved. However, for the time being, these cases have been omitted from the analysis of the results shown in the Table. It is felt at present that the optimal time for reading falls between 24 and 36 hours after the administration.

As can be seen in the Table, the skin test was strongly or weakly positive in all the volunteers previously exposed to IH virus who had shown clinical or laboratory signs of disease and also in 8 out of 9 of those who had failed to develop signs of infection, presumably because they were immune at the time of exposure. The one negative skin test was obtained in an individual who had been given a 10^{-6} dilution of IH serum containing possibly too little or no virus. In volunteers previously injected with SH virus the incidence of positive skin tests was much

lower, regardless of whether or not they had revealed clinical or laboratory signs of disease. Furthermore, the incidence of positive skin tests in a random group of adult volunteers at institution No. 1, previously not experimentally exposed to either IH or SH viruses, was similar to that encountered in the SH series at institution No. 2. On the other hand, only 1 out of the 9 children without a history of recognized hepatitis gave positive tests. On further investigation this child was found to have suffered 6 months previously of an illness clinically compatible with hepatitis without jaundice at an institution where IH was prevalent. Each of the 10 patients convalescent of a recent attack of spontaneous IH showed positive skin tests.

These encouraging results are indicative of a specific test in that a difference in response of IH and SH patients is apparent. It is obvious, however, that the studies have to be enlarged and extended to other conditions of liver illness and other infectious diseases. Such studies are in progress.

The fact that approximately 39% of the adult individuals in the SH and control series gave positive tests with IH antigen may be an indication of past unrecognized infection with this agent. In the absence of reliable histories this point cannot be ascertained. However, that the positive skin test possibly denotes

immunity and the negative results susceptibility may perhaps be gleaned from a comparison of the incidence of experimental IH in the groups of volunteers and the results of the skin test in a similar group chosen at random in institution No. 1. Over a period of 2 years 54 volunteers were exposed and 34 developed clinical or laboratory signs of hepatitis, 8 with jaundice; *i.e.*, 63% were definitely susceptible. Of individuals with specific skin test responses, 33 or 59% were negative and presumably susceptible. Although this appears to be a striking agreement this point requires confirmation by studying the effect

of natural or experimental exposure to IH virus in individuals with negative and positive skin tests, respectively.

Summary. A skin test antigen for infectious hepatitis (IH) has been prepared from amniotic fluid of chick embryos infected with IH virus. Thus far, it has given apparently specifically positive skin reactions in all the individuals with past histories of spontaneous or induced IH tested. In contrast, the incidence of positive tests among cases of past serum hepatitis was no greater than that found among an adult group chosen at random.

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***In Vitro* Histamine Release from Sensitized Rabbit Blood Cells. Evidence Against Participation of Fibrinolysin. (17759)**

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A considerable amount of circumstantial evidence has been presented to support the view that the release of histamine associated with anaphylaxis depends upon a proteolytic mechanism. The evidence has been obtained from studies in the dog, guinea pig, and rabbit and some of the studies appear to implicate the plasma protease, fibrinolysin (1-7). The evidence cited is indirect and circumstantial and a more direct type of evidence is desirable.

In this paper are presented the results of our studies on the question of whether fibrinolysin participates in the release of

histamine from the blood cells of the sensitized rabbit by antigen, *in vitro*. The availability of (a) crystalline soy bean trypsin inhibitor(8), which is a potent inhibitor of fibrinolysin, and (b) purified bovine fibrinolysin and antifibrinolysin(9,10) offer a direct experimental approach to this problem.

Experimental. The details of rabbit sensitization, exsanguination, the *in-vitro* histamine release reaction, and histamine determination were presented previously(11). Silicone-coated glassware was used for the handling of whole blood and for the histamine release reaction. The authors are grateful to Dr. M. Kunitz for samples of crystalline trypsin and soy bean trypsin inhibitor, STI, and to Eugene C. Loomis for samples of purified bovine fibrinolysin and antifibrinolysin. Some of the STI used was prepared in our own laboratory by the method of Kunitz.

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TABLE I.
Failure of STI to Inhibit Histamine Release from Sensitized Rabbit Blood Cells by Antigen.

Rabbit No.	Histamine in plasma, μg per ml of blood				
	No antigen	Antigen	STI and antigen	STI level, mg/ml	Inhibition by STI, %
900	.20	1.0	1.1	.4	-12
901	.22	0.78	0.87	.4	-16
7	.05	1.4	1.1	.8	21
8	.13	1.6	2.0	.8	-27
13	.14	2.2	2.4	.8	-9
14	.10	1.7	1.6	.8	6
19	.13	2.6	1.9	1.6	28
20	.08	0.88	0.88	1.6	0
37	.05	1.1	1.1	1.6	0
38	.11	1.9	1.9	1.6	0

Studies on the rate of histamine release were carried out as follows: Blood and saline solutions were placed in a 37°C water bath and were allowed 15 minutes to reach the temperature of the bath; 25 ml of blood was mixed with 18.8 ml of saline containing (a) no STI and (b) 100 mg of STI. After 5 minutes 12.5 ml of 1:100 egg white in saline was added rapidly with thorough mixing. At 1, 2, 3, 5, and 10 minutes after the addition of antigen, duplicate 4.5 ml aliquots were transferred to conical centrifuge tubes in an ice bath, each tube containing 1 ml of 0.1 M sodium oxalate. The oxalate was used to stop the histamine release reaction immediately(11). The contents of the centrifuge tubes were mixed thoroughly, chilled a few minutes, and centrifuged. The diluted plasma samples were then carried through the histamine purification and determination. The controls on these experiments were blanks at 0 time and 10 minutes, and histamine release by trypsin in the presence and in the absence of STI.

Experiments on the histamine release from normal blood by antigen and plasma from a sensitized animal were performed as follows: To 0.1 ml of blood plasma from a sensitized rabbit either 2.3 ml of saline or 1.3 ml of saline and 1 ml of STI solution (8 mg of STI per ml in saline) were added. One ml of 1:100 egg white in saline was added and the solution was placed in a 37°C water bath for 30 minutes. Two ml of normal rabbit blood at 37° was then added with thorough mixing; the tube was kept at 37° for an additional 10 minutes and then placed in an ice bath. The blood cells were

removed by centrifugation and the plasma was analyzed for histamine. The data reported are the averages of duplicate samples. Duplicates ordinarily agreed within $\pm 10\%$.

Results and discussion. Our data indicate that the anaphylactic release of histamine from rabbit blood cells *in vitro* does not depend upon the activation of the plasma protease, fibrinolysin. The data of Table I show that a high level of soy bean trypsin inhibitor (STI) fails to inhibit appreciably the release of histamine when antigen is added to blood from the sensitized rabbit *in vitro*. The amount of STI which failed to inhibit the histamine release by antigen (the highest concentration used) is 4 times the quantity which gave a nearly complete inhibition of histamine release by an optimal concentration of trypsin in the majority of cases (Tables II, III, and IV). This is approximately 80 times the amount of STI used by Gerheim and Ferguson(12) to inhibit the fibrinolysin in staphylokinase-activated rabbit plasma.

Plasma samples from the reactions where STI failed to inhibit histamine release by antigen, and from parallel samples without STI, were dialyzed and concentrated and tested for their ability to inhibit histamine release from rabbit blood cells by trypsin. The results (Table II) indicate that after the failure of STI to inhibit histamine release by antigen there is sufficient STI remaining in the plasma to inhibit markedly the histamine release by trypsin. In the

12. Gerheim, E. B., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, v71, 201.

TABLE II.
Inhibition of Trypsin Histamine Release by STI Remaining in Plasma After Failure to Inhibit Histamine Release by Antigen.

Plasma No. from Table I†	% inhibition of trypsin histamine release*		
	Plasma from reaction without STI	Plasma from reaction with STI	Control STI 0.4 mg/ml
19	2	26	34
20	— 2	36	34
37			
1	21	63	98
2	20	91	99
38			
1	16	95	98
2	53	82	99

* Trypsin conc. was 0.15 mg/ml.

† Plasmas No. 37 and 38 were tested for STI with trypsin reactions on each of two different normal rabbit bloods. Plasmas No. 19 and 20 were tested on a different date and with only one trypsin reaction.

TABLE III.
Histamine Release from Normal Blood Cells by Sensitized Plasma and Antigen.

	Histamine in plasma, μ g per ml of blood			
	Rabbit No. 1	Rabbit No. 2	Rabbit No. 3	Rabbit No. 4
Blood + Saline	0.18	0.23	0.21	0.41
" + SP + antigen	1.6	0.94	3.1	2.8
" + " + STI + antigen	1.5	0.98	2.9	2.7
" + trypsin	2.3	1.8	4.1	3.7
" + STI + trypsin	0.29	0.23	0.21	0.38

TABLE IV.
Rate of Histamine Release by Antigen in Presence and in the Absence of STI.*

Reaction time, min.	Histamine in plasma, μ g per ml of blood							
	Rabbit No. 233		Rabbit No. 234		Rabbit No. 317		Rabbit No. 318	
	No STI	STI	No STI	STI	No STI	STI	No STI	STI
	With antigen							
1	.58	.62	.24	.28	.26	.43	.16	.20
2	1.3	1.5	.64	.50	.44	.58	.31	.30
3	3.3	3.2	1.9	1.6	.93	.93	.74	.71
5	4.7	5.2	2.6	2.2	1.4	1.5	1.1	1.3
10	6.1	5.4	3.2	3.2	1.8	2.1	1.8	1.4
	Without antigen							
0	—	—	—	—	—	—	.35	—
10	.58	—	.31	—	.42	—	.56	—
	With trypsin							
10	5.0†	1.9†	2.8†	1.0†	2.9	.38	1.80	.41

* Conc. of STI was 1.6 mg/ml in final dilution, or 4 mg/ml of blood in the reactions with antigen; the STI level in the trypsin reactions was 0.8 mg/ml in the final dilution.

† In these experiments the trypsin controls were in the 37° bath for 20 min.

presence of this excess of STI, it is difficult to believe that the plasma protease could in any way participate in the histamine release.

As an answer to this evidence the explana-

tion has been offered that fibrinolysin might release the histamine immediately following or simultaneously with its activation, before STI can inhibit the reaction. To test this

TABLE V.
Histamine Release from Rabbit Blood Cells by Bovine Fibrinolysin, Trypsin, and Antigen.

Rabbit No.	Enzyme conc., mg per ml		Histamine in plasma, μg per ml of blood			
	Fibrinolysin	Trypsin	Controls	With fibrinolysin	With trypsin	With antigen
5	1.6	.10	.12	.37	1.7	—
6	1.6	.10	.11	.14	1.8	—
7	3.2	.15	.05	1.7	2.7	1.4
	4.8			1.5		
8	3.2	.15	.13	.26	2.3	1.6
	4.8			1.5		
13	4.	.15	.14	.28	2.6	2.2
14	4.	.15	.10	.80	2.2	1.7
995	8.	.15	.10	.39	1.8	2.5
996	8.	.10	.05	.38	1.3	.9

TABLE VI.
Effect of Bovine Antifibrinolysin on Histamine Release by Antigen.

Rabbit No.	Histamine released,* μg per ml of blood		Inhibition by antifibrinolysin, %
	No antifibrinolysin	Antifibrinolysin	
217	2.2	2.8	—27
218	2.4	2.8	—17
219	3.9	4.1	—5
220	3.0	2.3	23

* Histamine released = (histamine in plasma after antigen reaction — histamine in plasma blanks.)

possibility we studied the release of histamine from normal rabbit blood by means of "sensitized plasma" and antigen. As shown in Table III a good release of histamine from rabbit blood cells was obtained when antigen and sensitized plasma were allowed to react for 30 minutes at 37°C prior to their mixing with normal blood. Again the histamine release was not affected by the presence of a high level of STI. Inasmuch as the histamine release from sensitized blood cells is nearly complete in 5 minutes at 37°C (Table IV), the histamine release mechanism must be activated quite rapidly. The 30 minute period at 37° in which sensitized plasma, antigen, and STI are allowed to react should be ample time for any active fibrinolysin to become inactivated by STI. The failure of STI to inhibit the histamine release under these conditions is strong evidence that fibrinolysin has no significant role in the histamine release mechanism under investigation.

Scroggie, *et al.* (13) showed that STI delays

the release of histamine from rabbit blood cells by peptone. Our data on the rate of histamine release by antigen in the presence of and in the absence of STI, Table IV, indicate that STI has no effect upon the rate of the anaphylactic release of histamine. This may mean that the mechanisms of peptone and anaphylactic histamine release are dissimilar.

Additional evidence against the participation of fibrinolysin in the *in vitro* anaphylactic histamine release is: (1) the addition of high levels of bovine fibrinolysin to rabbit blood gave a poor and unreproducible release of histamine as compared to the amount released by much lower concentrations of trypsin and by antigen, Table V. The concentrations of fibrinolysin shown in Table V represent the final dilution in the reaction. The highest level used would be 20 mg per ml of blood—an extremely high concentra-

13. Scroggie, A. E., Jaques, L. B., and Rocha e Silva, M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 326.

tion even through the enzyme may not be 100% pure. (2) Bovine antifibrinolysin, 5 mg per ml of blood, failed to inhibit the histamine release by antigen as shown by the data of Table VI.

The evidence presented here applies only to the mechanism of histamine release from rabbit blood cells by antigen *in vitro* and may have no bearing upon the mechanism of histamine release by antigen in other species. However, in accord with our results is the recent work by Scroggie and Jaques (14), which indicates that in the dog the activated plasma protease is not necessary for the anaphylactic release of histamine in the liver.

The above evidence indicates that any

14. Scroggie, A. E., and Jaques, L. B., *J. Immunol.*, 1949, v62, 103.

activation of profibrinolysin or any increase in plasma protease activity, which may accompany the *in vitro* histamine release by antigen, is only a coincidental phenomenon and is not a prerequisite for the histamine release.

Summary. The failure of excessive concentrations of soy bean trypsin inhibitor and bovine antifibrinolysin to inhibit the histamine release by antigen and the poor and inconsistent histamine release by bovine fibrinolysin indicate that the *in vitro* anaphylactic release of histamine from rabbit blood cells does not depend upon fibrinolysin activity.

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Release of Adrenocorticotrophic Hormone by Direct Application of Epinephrine to Pituitary Grafts.* (17760)

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It is now known that the injection of epinephrine is followed in normal rats by a fall in the adrenal ascorbic acid(1) and in circulating blood eosinophiles(2). Long(3) has suggested that in many of the conditions associated with an increased degree of activity of the pituitary-adrenal system, a preliminary release of epinephrine is a contributing factor. On the other hand, Sayers and Sayers(4) have found that there is an inverse relationship between the blood levels of adrenocorticotrophic hormone (ACTH) and adrenal corti-

cal hormone (ACH) that determines the relative secretory activity of each gland. They regard the effect of epinephrine as due to its capacity to increase the utilization of cortical hormone and thus reduce its level in the blood traversing the pituitary.

In order to determine whether epinephrine is capable of directly increasing the secretion of ACTH from the anterior pituitary, the following experiment was performed. A group of adult, male, albino rats of the Sprague-Dawley strain, weighing about 250 g each, were hypophysectomized by the parapharyngeal approach, and 3 days later homologous transplants of pituitary tissue from female adult rats of the same strain were inserted into the anterior chamber of the right eyes. Seven rats survived the operations and 6 weeks later all had well vascularized grafts growing in the anterior chamber of the eye. The eosinophile decline in the peripheral blood was selected as an index of adrenal cortical activity

* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

1. Long, C. N. H., and Fry, E. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, v59, 67.

2. Recant, L., Forsham, P. H., and Thorn, G. W., *Fed. Proc.*, 1948, v7, 99.

3. Long, C. N. H., *Fed. Proc.*, 1947, v6, 461.

4. Sayers, G., and Sayers, M., *Recent Progress in Hormone Research*. Vol. II. Academic Press, New York, 1948.

and hence of ACTH release from the grafts. The subcutaneous injection of epinephrine (.02 mg/100 g) brought about a definite eosinopenic response in three hours, the mean fall being $-46 (\pm 3)\%$. Previous studies had demonstrated that a series of hypophysectomized rats given a similar dose of epinephrine showed a mean rise of $+20 (\pm 8)\%$.

In order to test the effects of the local application of epinephrine directly on pituitary tissue, under light Nembutal anesthesia a small slit was made with a scalpel in the sclero-corneal junction and .05 cc of a 1-250,000 solution of epinephrine was instilled directly into the anterior chambers with a fine needle. All 7 rats showed a definite eosinopenic response in three hours, with a mean fall of $-44 (\pm 2)\%$.

An interval of 3-5 days was allowed to elapse between successive experiments on any given rat and then an identical procedure was carried out, using the same dose and concentration of epinephrine in the normal eyes. None of the rats showed any significant change in eosinophile count over a period of 3 hours, the mean variation being $+6 (\pm 4)\%$.

Since it was of interest to determine if endogenous epinephrine released from the adrenal medulla would activate the grafted pituitary tissue each rat was given—as a painful stimulus which would not produce any gross physiological stress or extensive tissue damage—a subcutaneous injection of 1.0 cc of hypertonic (10%) saline. All animals showed eosinopenic responses in 3 hours, with a mean fall of $-40 (\pm 5)\%$.

As a final experiment on this series of 7 animals, the eyes containing the grafts were enucleated, and 3-5 days following enucleation each rat was given another subcutaneous dose of epinephrine (.02 mg/100 g). Following removal of the grafted pituitary, none of the animals showed an eosinopenic change. In fact, the mean change was a rise of $+13 (\pm 6)\%$, comparable to the rise of $+20 (\pm 8)\%$ noted in a series of eight non-grafted hypophysectomized rats.

One of the grafts was assayed for ACTH by grinding the fresh tissue with 1.0 cc of isotonic saline containing 2 drops of .1 N NaOH.

This extract was then injected intravenously into each of two hypophysectomized rats in the amount of 0.1 cc/100 g. Both rats showed eosinopenic responses (mean -54%) and a fall in total ascorbic acid (mean -28%).

It is of interest to note that the adrenal glands of the hypophysectomized rats with homologous pituitary grafts averaged 10.3 mg/100 g in weight, while the adrenal glands of non-grafted hypophysectomized rats, after a corresponding time interval following operation, averaged 5.7 mg/100 g in weight. The weights of adrenal glands of normal rats in our studies have averaged 14.0 mg/100 g. It would appear then that not only do these grafts have the ability to release ACTH in response to stimulation by systemic or local epinephrine, but also release steadily enough ACTH to support, at least partially, the weight of the adrenal glands. There is no evidence at the moment that these grafts release either growth hormone or gonadotrophic hormone since the animals did not grow and the testes were completely atrophic.

This experiment, taken in conjunction with those recently reported by Cheng *et al.*(5) and Fortier and Selye(6), would indicate that no neural or neurohumoral connections with the central nervous system are necessary for the release of ACTH from the anterior lobe of the pituitary.

Summary. 1. Homologous grafts of pituitary tissue to the anterior chamber of the eye of the hypophysectomized rat retain the ability to secrete ACTH, both spontaneously and in response to activation by epinephrine.

2. The effect of epinephrine in releasing ACTH may be brought about by its direct application to anterior pituitary tissue.

The authors are much indebted to Dr. H. S. N. Greene for advice and assistance in the preparation of the homologous pituitary grafts.

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Influence of Cold, Fasting and Adrenalectomy on the Blood-Fibrinogen Response to Trauma. (19761)

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A reduction of the plasma fibrinogen in fasting rats exposed to cold has been previously reported(1). On the other hand a rise in plasma fibrinogen is observed after surgical trauma(2) and a wide variety of physical and chemical injuries(3,4). Hence it appeared important to determine whether cold and fasting would reverse the blood-fibrinogen rise normally induced by trauma.

As during exposure to cold important changes occur in the adrenals(5), we also studied the influence of adrenalectomy upon the fibrinogen response to injury.

Exp. I. Influence of fasting on the fibrinogen response to trauma. 74 female, piebald rats, weighing from 130 to 180 g were distributed into 8 groups. Group I served as absolute controls. Groups II, III and IV were fasted for periods of 24, 48 and 72 hours respectively, before killing. Groups V and VI were submitted to complete nephrectomy, the former being normally fed (Purina Fox Chow) and the latter fasted for a period of 48 hours. The operation was performed after 24 hours

of fasting. Groups VII and VIII were *partial*-ly nephrectomized, the latter being fasted for a total of 72 hours (48 hours before and 24 hours after the operation).

All operated animals were bled and killed 24 hours after the surgical trauma and the remainder at the end of the periods indicated above.

Plasma fibrinogen determinations in this and in the subsequent experiments were made by a modification of the method of Campbell and Hanna(1).

As can be seen from Table I, periods of 24, 48 and 72 hours fasting depress constantly, though not always significantly, the blood-fibrinogen concentration of intact animals.

It is also shown that the plasma fibrinogen response to the trauma of nephrectomy is unaffected by fasting periods of 48 and 72 hours.

Exp. II. Influence of cold on the fibrinogen response to trauma. 47 female piebald rats, weighing from 130 to 170 g were divided into 5 groups. Group I served as absolute controls. Group II was unilaterally nephrecto-

TABLE I.
Influence of Fasting on Fibrinogen Response to Trauma.

Groups	Treatment	No. of animals	Body wt		Fibrinogen mg/100 ml
			Initial	Final	
I	Normal control	8	145 ± 10*	-	252 ± 13†
II	24 hr fasting	9	140 ± 10	130 ± 13*	231 ± 12
III	48 " "	8	144 ± 11	124 ± 11	203 ± 12
IV	72 " "	11	166 ± 9	136 ± 7	229 ± 9
V	24 " after complete nephrectomy	9	141 ± 7	149 ± 12	540 ± 26
VI	Complete nephrectomy 48 hr fasting	6	147 ± 12	133 ± 12	516 ± 21
VII	24 hr after partial nephrectomy	9	141 ± 7	149 ± 12	449 ± 11
VIII	Partial nephrectomy 72 hr fasting	12	166 ± 9	135 ± 8	484 ± 23

* Standard deviation.

† Standard error.

* Instituto Butantan, Sao Paulo, Brazil.

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4. Chanutin, A., and Ludewig, S., *J. Biol. Chem.*, 1947, v167, 313.

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TABLE II.
Influence of Cold on Fibrinogen Response to Trauma.

Groups	Treatment	No. of animals	Body wt		Fibrinogen mg/100 ml
			Initial	Final	
I	Normal control	10	-	149 ± 3*	243 ± 8.9†
II	24 hr cold after unilateral nephrectomy	12	153 ± 7*	146 ± 6	470 ± 12
III	24 hr cold + fast after unilateral nephrectomy	8	155 ± 7	127 ± 4	373 ± 13
IV	24 hr cold -	9	146 ± 10	155 ± 14	244 ± 10
V	24 hr fast cold	8	147 ± 12	129 ± 13	190 ± 14

* Standard deviation.

† Standard error.

TABLE III.
Influence of Adrenalectomy on Fibrinogen Response to Trauma.

Groups	Treatment	No. of animals	Body wt		Fibrinogen mg/100 ml
			Initial	Final	
I	Intact control	7	168 ± 9*	180 ± 12*	243 ± 9†
II	Adr-X + 25 days rest then unilateral nephrectomy	10	168 ± 17	176 ± 23	420 ± 22
III	Adr-X + 25 days rest	9	166 ± 11	176 ± 22	346 ± 24
IV	Adr-Xp + 25 " "	5	170 ± 10	184 ± 14	243 ± 27
V	Adr-Xp + 25 " " then unilateral nephrectomy	6	165 ± 12	176 ± 11	534 ± 26

* Standard deviation.

† Standard error.

Adr-X Complete adrenalectomy.

Adr-Xp Unilateral adrenalectomy.

mized and following operation was exposed to cold for 24 hours (1 to 5°C). Group III was similar to II but in addition was fasted during the exposure interval. Groups IV and V served as controls for cold and cold combined with fasting respectively.

Table II summarizes the results. It is to be seen that although fibrinogen is not altered by exposure to cold alone, it is markedly decreased under the combined influence of fasting and cold. On the other hand it can be seen from Experiment II that the blood-fibrinogen is only slightly diminished by fasting alone.

As regards the operated animals, it is seen that addition of fasting to exposure to cold reduces significantly the fibrinogen response to trauma.

Exp. III. Influence of adrenalectomy on the fibrinogen response to trauma. 37 piebald female rats, weighing from 140 to 200 g were distributed into 5 groups. Group I served as intact control. Groups II and III were totally

adrenalectomized. Groups IV and V were unilaterally adrenalectomized. In order to control for the trauma of total adrenalectomy, a laparotomy was performed on the opposite side, although the remaining adrenal was left intact.

All animals were kept on Purina Fox Chow and an aqueous solution containing 0.97% of sodium chloride and 0.03% of sodium bicarbonate in place of drinking water, for the duration of the experiment. The adrenalectomized rats were in good condition and even gained some weight, during the experimental period. Twenty-five days after the beginning of the experiment, Groups II and V were unilaterally nephrectomized. The following day all the animals in all groups were bled and killed. Autopsy failed to reveal either detectable infection in any group or adrenal remnants in the adrenalectomized groups.

The results are summarized in Table III. It is to be noted that total adrenalectomy greatly decreases, but does not abolish, the

fibrinogen response to trauma.

It is shown that totally adrenalectomized animals, 25 days after the operation still have a blood-fibrinogen concentration higher than intact controls, while rats unilaterally adrenalectomized at the end of the same period already have normal values.

Thus total adrenalectomy while diminishing the fibrinogen response to trauma delays its return to normal values.

Discussion. The data for plasma fibrinogen of fasted non-operated animals indicate a certain correlation between the decrease in fibrinogen and in body weight. This confirms previous experiments on dogs(6) and shows indirectly the relation between increased catabolism and decrease in fibrinogen. Incidentally we should like to mention that plasma proteins are considered to be freely utilized by tissues during emergencies(7,8).

Simultaneous exposure to cold and fasting for 24 hours, while causing a greater decrease in fibrinogen, induced approximately the same loss in weight as fasting for 48 hours. However, it is to be noted that in the first case the same loss of weight occurred during a shorter period, thus indicating a greater rate of catabolism. This finding could explain the sharper decrease in fibrinogen in the first instance. The same relation between fibrinogen decrease and weight loss appears to exist after injury in animals exposed to cold and fasting.

Exposure to cold is followed by an increased production of certain hormones such as corticotrophin(9), corticoids(10), thyroid hormone(11) and epinephrine(12). None of these is known to cause any marked decrease

in blood-fibrinogen. It was observed that bovine corticotrophine causes a marked increase in plasma fibrinogen while ovine corticotrophine induces a milder rise(13), this suggests a foreign protein effect(14). Preliminary experiments with prolonged thyroxine treatment did not show any influence upon plasma fibrinogen(13) while epinephrine increased it markedly(13,15).

Our experiments with adrenalectomized rats suggest that the fibrinogen metabolism is influenced by the adrenals. The delayed return of plasma fibrinogen to normal after adrenalectomy could be explained by the diminished capacity of the adrenalectomized rat to catabolize protein(16). The smaller response to a second trauma indicates a difficulty in the synthesis of new protein in the absence of the adrenals. Further work is necessary to elucidate whether this fact is dependent upon a deficiency in corticoids.

Summary. Periods of 24, 48 and 72 hours of fasting constantly, though not always significantly, depress the blood-fibrinogen concentration in intact rats.

The blood-fibrinogen response to trauma is unaffected by fasting periods of 48 and 72 hours.

Simultaneous exposure to cold and fasting significantly reduces the fibrinogen rise induced by trauma.

Total adrenalectomy greatly decreases but does not abolish the fibrinogen response to injury. However, the blood-fibrinogen increase following an operation is prolonged in totally adrenalectomized as compared to partially adrenalectomized rats.

These observations suggest that the adrenals play an important role in fibrinogen metabolism.

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Inactivation of Several Viruses by Liquid Ethylene Oxide. (17762)

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Sterilization of bacteriological media, as well as materials such as serum and milk, by liquid ethylene oxide has been recently demonstrated by Wilson and Bruno(1) to be a simple and efficient laboratory procedure. Not only is ethylene oxide able to kill a number of aerobic gram positive and gram negative organisms as well as aerobic and anaerobic spore-forming bacilli, but also the media can be readily rid of the sterilizing agent, and then once more be capable of supporting bacterial multiplication(1). Phillips and Kaye recently reviewed the literature on the sterilizing action of gaseous ethylene oxide and pointed out the wide spectrum of microorganisms for which it is lethal(2).

Following the demonstration(1) that 1% liquid ethylene oxide can inactivate vaccinia virus, it was of interest to determine whether this viral sterilizing property could be extended to a variety of other viruses. It is the purpose of this paper to report that viruses of the influenza-Newcastle disease group as well as neurotropic viruses of the mouse encephalomyelitis type are readily inactivated by this liquid chemical sterilizing agent.

Materials and methods. Viruses. The following viruses were employed: influenza A, PR8 strain; influenza B, Lee strain; Newcastle disease virus, Beaudette strain; Columbia MM mouse encephalomyelitis virus; Theiler's mouse encephalomyelitis virus, FA strain. These viruses shall be referred to as PR8, Lee, NDV, Col. MM, and FA, respectively. PR8, Lee and NDV were cultivated in the allantoic sacs of 9-11-day-old chick embryos. The PR8 and Lee viruses were stored between passages as infected allantoic fluid, and NDV was stored as a 10^{-1} dilution of infected allantoic fluid in

heated normal horse serum. The Col. MM and FA viruses were cultivated by intracerebral inoculation of The Rockefeller Institute strain of albino Swiss mice, and stored as 10% mouse brain suspensions in 0.85% NaCl buffered at pH 7.2 (0.01 M phosphate). All virus suspensions were stored in a solid carbon dioxide cabinet at -70°C .

Virus Infectivity and Hemagglutination Titrations. The methods employed with Lee, PR8 and NDV viruses were carried out as previously described(1). Serial 10-fold dilutions of infected allantoic fluid were made in broth, and appropriate dilutions were inoculated into the allantoic sacs of chick embryos 9-11 days of age. Four chick embryos per dilution were employed. Following incubation for 2 days at 35°C and subsequent chilling, the allantoic fluid of each embryo was removed. Hemagglutination titrations with 1% chicken erythrocytes were carried out through a dilution of 1.128 to determine the presence of viral infection. The 50% infectivity titer was calculated by the method of Reed and Muench(3). Infectivity titrations with Col. MM and Theiler's FA viruses were carried out by intracerebral inoculation of 10-fold dilutions of infected mouse brain; 0.03 cc per mouse. Groups of 6 mice per dilution were employed. The 50% mortality titer(3) was determined at the end of 14 days in each instance.

Experimental. Inactivation of Viruses with Ethylene Oxide. The ethylene oxide employed and the technics of handling it were identical to those previously described in detail(1). The respective viruses were diluted 10-fold in sterile, heat-inactivated normal horse serum, and the mixtures placed at 4°C for 60 minutes. Then, to a 9.9 cc aliquot of each virus-serum suspension was added 0.1 cc of liquid ethylene oxide with a chilled pipette, and the mixture agitated to insure thorough mixing. Follow-

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TABLE I.
Inactivation of Newcastle Disease Virus by Ethylene Oxide.

Virus*	Ethylene oxide, vol. %	Mixture held		Virus dilution inoculated intra-allantoic (.1 cc/embryo)	Hemagglutination titer† of allantoic fluids‡				Score +/t§	Infectivity titer E.I. 50
		at 4°C, hr	at 35°C, hr		1	2	3	4		
NDV 10 ²	0	1	24	10 ⁻⁶ 10 ⁻⁷ 10 ⁻⁸	>128 >128 >128	>128 >128 0	>128 >128 >128	>128 >128 0	4/4 4/4 2/4	10 ⁻⁸ or >
"	1	1	24	10 ⁻² 10 ³	0 0	0 0	0 0	0 0	0/4 0/4	<10 ⁻²

* Suspended in 99% normal horse serum.

† Expressed as the reciprocal.

‡ Harvested from embryos after incubation at 35°C for 48 hr.

§ Numerator represents the number of chick embryos infected; denominator represents the number of chick embryos inoculated.

|| E.I. 50 = 50% embryo infectivity titer.

ing a further interval of 60 minutes at 4°C, the virus-ethylene oxide mixtures were transferred to an incubator regulated at 35°C where they remained for 24 hours. During this period of incubation the virus was acted upon by the sterilizing agent, and the agent itself disappeared by volatilization and combination. A second aliquot of each virus suspension was employed as a control, and carried through identical steps excluding the addition of ethylene oxide. Following the 24 hours incubation period at 35°C, infectivity titrations were carried out in appropriate hosts.

Inactivation of the Viruses of Influenza A and B and Newcastle Disease. In each instance liquid ethylene oxide in a concentration of 1 volume % was capable of causing complete inactivation of the virus as determined by the ability of the virus to infect the chick embryo. The results of a typical experiment employing NDV are shown in detail in Table I. It will be noted that even when more than 10⁶ embryo infectious doses of virus was present initially, not a single embryo was infected when inoculated with the virus-ethylene oxide mixture. The results of experiments with NDV, PR8 and Lee are summarized in Table II, indicating the effectiveness of ethylene oxide in rendering these three distinctly different viruses non-infectious. It is of considerable interest and importance that dilution of the virus suspensions in 90-99% normal horse serum did not diminish the effectiveness of ethylene oxide. That various animal sera have a protective effect upon viruses in the presence of many inactivating substances as well as pH alterations is well known. Inactivation of even larger quantities of viruses by ethylene oxide could not be tested readily because of the necessity of incubating the mixtures for 24 hours at 35°C as part of the inactivation procedure. Incubation alone, in the absence of ethylene oxide, resulted in some viral inactivation in each instance.

Inactivation of Neurotropic Viruses. The infectivity of mouse encephalomyelitis viruses, Columbia MM and Theiler's FA, when suspended in normal horse serum was likewise completely inactivated by 1% liquid ethylene

TABLE II.
Inactivation of Various Viruses by Ethylene Oxide.

Virus	Ethylene oxide, %	Infectivity titer, E.I. 50*	Quantity virus inactivated, E.I. 50
Influenza B	0	10-5.7	
	1	<10-1	104.7 or >
Influenza A	0	10-4	
	1	<10-1	103 or >
Newcastle disease	0	10-8	
	1	<10-2	106 or >
		M.L. 50†	M.L. 50
Theiler's Fa	0	10-4.6	
	1	<10-2	102.6 or >
Col. MM	0	>10-6.4	
	1	<10-2	104.4 or >

* E.I. 50 = 50% embryo infectivity.

† M.L. 50 = 50% mouse mortality.

oxide. The results of these experiments are summarized also in Table II. It should be emphasized that neither with Col. MM nor FA did a single mouse inoculated intracerebrally with the ethylene oxide-treated virus show any evidence of infection by the neurotropic virus. Eighteen days following inoculation with the inactivated virus, the mice were challenged with 10^2 M.L.50 doses of the homologous active virus, and no evidence of immunity could be demonstrated in either instance.

Discussion. That liquid ethylene oxide can inactivate a variety of unrelated viruses is clearly indicated by the experimental data presented. Similar inactivation of the vaccinia virus also has been reported(1). That such viral inactivation can be accomplished with considerable reliability, ease, economy and safety is apparent. Viral inactivation was so complete, even in the case of NDV where more than 10^6 E.I.D. of virus was present, that not a single animal, chick embryo or mouse was infected. This is of particular significance with viruses tested in the chick embryo in which, in the case of the influenza viruses, infection is produced by about 10 viral particles or less(4). It is of considerable

interest and importance that the ethylene oxide is active in the presence of 90-99% serum but causes no visible change in the serum. That some chemical alterations of the proteins do take place seems probable, but whether these changes affect the physiological activities of the serum proteins is not known. It has been clearly demonstrated(1), however, that the growth factors of bacteriological media are not sufficiently modified to prevent the subsequent growth and multiplication of fastidious bacteria in these media. The possibility is raised that the virus of homologous serum hepatitis in plasma, serum or other blood products might be inactivated by this procedure. Were this the case, it would indeed afford a simple and practical method of ridding these important therapeutic substances of a stable, troublesome, and dangerous virus.

Summary. Liquid ethylene oxide in a concentration of 1 volume % inactivates the viruses of influenza A, influenza B, and Newcastle disease as well as Columbia MM and Theiler's FA mouse encephalomyelitis viruses. These viruses were inactivated in the presence of 90-99% normal horse serum without any gross alterations in the serum.

4. Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, v79, 301.

Protective Effect of Cattle Sera on White Mice Infected with *Brucella abortus* with the Aid of the Mucin Technic. (17763)

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Dishon and Olitzki(1) produced lethal *Brucella*-infections in white mice with the aid of the mucin technic. The lethal effect of intraabdominal injection of 0.5 cc of a broth culture of the test organism together with 0.5 cc of a 10% mucin suspension was irregular. Since it is difficult to inject more than 1.0 cc of this mixture, the production of regular lethal infections with the aid of concentrated broth cultures has been studied. The experiments showed that injection of 0.5 cc of a 4-fold concentrated 48 hour broth culture together with 0.5 cc of a 10% suspension of mucin regularly produces a lethal infection.

Mucin preparations manufactured by the Armour Laboratories, London, and Winthrop Stearns Inc., New York, were equally effective. To avoid dispersal of the highly infectious material in the laboratory, the *Brucellae* were cultured in centrifuge tubes containing 10 cc of broth. After incubation at 37°C for 48 hours, 3 out of 4 tubes were centrifuged, the supernatant fluids discarded and the sedimented bacteria suspended in the fourth broth culture. The bacterial suspension concentrated in 10 cc of broth contained the growth of 40 cc. After the addition of an equal quantity of the 10% mucin suspension 1.0 cc of the resulting mixture administered intraabdominally produced 100% mortality in white mice.

The protective effect of cattle sera. Sera were kindly provided by Dr. van der Hoeden, Director of the Central State Veterinary Laboratory in Tel Aviv. They were derived from nonimmunized cattle presumed to have had contact with *Brucellae* and from cattle which had been immunized with living "Swiss strain 19". Part 1 of Table I shows the protecting effect of 26 sera. The sera were injected into the dorsal muscles of the mice immediately after

the lethal inoculation with the *B. abortus*-mucin suspension. Only sera with an agglutinin titre for *B. abortus* higher than 500 were protective. Moreover, the protective effect was not correlated with the quantity of serum injected. A serum with an agglutinin titre of 1000 administered in quantities of 0.5 and 0.2 cc did not protect, whereas 0.1, 0.05 and 0.02 cc of the same serum protected. In some cases, mice which received 0.5 cc of serum died before the control animals. In general these experiments suggest that the agglutinin titre and the protective effect of cattle sera show no clearcut correlation when the serum is administered after the infection has taken place.

In another series of experiments, cattle sera were injected 24 hours before inoculation with *B. abortus*. A close correlation between agglutinin titre and protective effect was observed. Part 2 of Table I shows that only 10% of the sera with agglutinin titres below 200 gave positive protection tests, while 94% of the sera with agglutinin titres of 200 and more gave positive protection tests.

Correlation between the protective effect and the agglutinin titre. In general sera with agglutinin titres higher than 200 protected the mice when given in quantities of 0.01 cc or more. Table II shows the protective effect of five sera administered in varying quantities 24 hours prior to the inoculation of *B. abortus*. There is no definite correlation between the protecting and agglutinating power of the sera. Three sera with an agglutinin titre of 1000 gave 100% protection at 0.05, 0.05 and 0.01 cc, two sera with an agglutinin titre of 500 gave 100% protection when administered in quantities of 0.02 and 0.05 cc. Thus the ratio between the minimal agglutinating quantity per cc and the minimal 100% protecting quantity varies between 1:10 and 1:50 with an average of 1:30. The doses of serum which give a 50% protection vary between

1. Dishon, T., and Olitzki, L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 698.

TABLE I.
Agglutinin titre and protecting effect of cattle sera injected immediately after or 24 hours prior to the inoculation of *B. abortus*.

Time of admin.	Titre of agglutinins below	No. of sera examined	No. of sera showing protecting effect after the injection of	
			0.5 cc	0.2 cc
Immediately after the inoculation	200	16	0	0
	200	1	0	0
	500	2	0	0
	1000	3	0	1
	2000	3	1	2
	10000	1	1	1
24 hr. prior to inoculation	200	20	2	2
	200	3	2	2
	500	3	3	3
	1000	1	1	1
	2000	6	6	6
	5000	1	1	1
	10000	3	3	3

TABLE II.
Agglutinin titres and quantitative protection tests carried out with 5 sera.

No. of serum	Titre of agglutinins	No. of deaths after administration of serum (cc)								
		0.5	0.2	0.1	0.05	0.02	0.01	0.005	0.002	0.001
1	1000	0/2	0/2	0/2	0/4	1/4	3/4	3/4	3/4	4/4
2	1000	0/2	0/2	0/2	0/4	1/4	2/4	3/4	3/4	4/4
3	1000	0/2	0/2	0/2	0/4	0/4	0/4	4/4	4/4	3/4
4	500	0/2	0/2	0/2	0/4	0/4	1/4	1/4	3/4	4/4
5	500	0/2	0/2	0/2	0/4	1/4	1/4	2/4	1/4	4/4

0.012 and 0.004 cc and the ratios between the minimal agglutinating quantity per cc and the 50% protecting doses vary between 1:12 and 1:2 with an average of 1:6. The results show that the agglutinating and protecting powers of cattle sera against *B. abortus* are not closely related. In all instances, however, the serum doses required for 100% or for 50% protection were higher than the minimal agglutinating serum dose.

The bactericidal effect in vivo. Fifty-nine surviving mice were sacrificed on different days between 2 and 4 weeks after the day of infection. None of these animals gave a positive blood culture. Eighteen showed general infections of the abdominal organs, 20 local infections of the spleen and 6 local infections of the liver. It is evident that the administra-

tion of serum had not prevented the establishment of the microorganisms in these organs.

Summary. Lethal infections of *B. abortus* may be produced regularly in mice with the aid of the mucin technic by the injection of 0.5 cc of a 4-fold concentrated 48 hours broth culture. Cattle sera with agglutinin titres for *B. abortus* of 200 or higher protect the mice against the infection, when at least 0.05 to 0.02 cc of serum is administered 24 hours prior to infection. The minimal protecting dose and the minimal agglutinating serum quantity per cc are not closely linked. The serum prevents general septicemia and death, but does not prevent the formation of infectious foci in the abdominal organs.

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Effect of Biliary Stimulants on Disappearance Rate of Intravenously Administered Cholesterol. (17764)

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This investigation studied the effects of biliary salts (Bilron-Lilly and sodium dehydrocholate), on the removal of intravenously administered cholesterol from the blood stream in rabbits. All of the animals were given 100 mg of cholesterol per kg intravenously. Plasma containing 3000 mg % of cholesterol or more, which was obtained from rabbits previously fed cholesterol, was used as the source of cholesterol according to the procedure employed by Horlick, Feldman and Katz(1). The rabbits were divided into 3 groups, one serving as the control, the others receiving either Bilron or sodium dehydrocholate in

amounts indicated in the table. This was administered at the same time as the hypercholesteremic and hyperlypemic plasma and repeat doses were given 24 and 48 hours later. Total and free serum cholesterols were determined by the Schoenheimer-Sperry(2) method on samples taken before the injection and at intervals of 15 minutes, 24, 48 and 72 hours later. As shown in the Table I no significant effect was noted on the disappearance rate of the injected cholesterol following the administration of the test drugs. Since these bile salts, especially Bilron, increase the 24 hour biliary cholesterol excretion it may be

TABLE I.

Control*		15 min		24 hr		48 hr		72 hr		Hematocrit fall§
Cholesterol Total	Free	Cholesterol Total	Free	Cholesterol Total	Free	Cholesterol Total	Free	Cholesterol Total	Free	
I. Sodium Dehydrocholate.†										
91	39	205	83	178	71	145	63	150	57	9
50	19	238	70	122	31	84	33	90	40	8
69	25	255	100	128	33	128	44	122	48	8
55	21	205	83	97	21	99	55	134	77	7
43	23	248	92	111	36	107	40	113	42	7
II. Bilron.‡										
66	44	263	68	68	44	Not taken		150	71	5
73	36	275	75	127	68			153	63	4
73	22	295	94	108	44	Expired 40 hrs				
81	40	256	88	104	48	109	59	159	75	
68	30	248	82	111	47	113	52	142	53	1
90	42	266	92	132	59	110	46	110	38	5
45	31	186	63	73	37	89	30	111	33	1
III. Control Rabbits.										
40	25	220	70	125	55	—	75	160	80	4
60	40	230	80	150	75	80	45	70	40	6
80	25	280	85	120	48	90	55	100	65	3
70	50	260	90	110	55	95	48	80	60	4
70	50	260	93	170	90	120	45	90	45	3

* Cholesterol (100 mg/kg) inj. intrav. together with test drug immediately after withdrawal of control sample.

† All rabbits given sodium dehydrocholate intrav. with equal repeat doses at 24 and 48 hr; total, 2 g.

‡ Rabbits No. 1-3 given Bilron (Lilly) by stomach tube, with equal repeat doses at 24 and 48 hrs; total, 2 g. Rabbits No. 4-7 same as † but total 2.6 g.

§ Fall in hematocrit between control and 72-hr samples.

concluded that the removal of intravenously administered cholesterol was not significantly accelerated by either this mechanism or by any possible more direct action of these sub-

stances on cholesterol metabolism.

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Platelet Regeneration During Therapy of Acute Leukemia with Folic Acid Antagonists.* (17765)

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Temporary remissions in acute leukemia have been produced by the therapeutic administration of the various folic acid antagonists. Observations have varied as to the response of the blood platelets. Farber *et al.*(1,2) reported improvement in the platelet level in some of his cases. Dameshek(3) in a preliminary report stated that a definite increase in blood platelets must be considered as a part of the criteria in determining whether or not a remission had occurred but no other data are given. Meyer *et al.*(4) observed no significant effect on the platelet level of patients with acute and chronic leukemia treated with aminopterin (4-amino, pteroyl glutamic acid). Berman *et al.*(5) reported no regular response in platelets in nine cases of chronic leukemia treated with aminopterin. Stickney *et al.*(6) merely state that the platelets may

increase in number during remissions. Sacks *et al.*(7) reported his findings in 14 cases of acute leukemia and in 4 cases with an initial thrombocytopenia a definite platelet response occurred.

In a series of 48 patients (children and adults) with acute leukemia of all types treated with folic acid antagonists, results were obtained which indicated that when a definite remission occurred the elevated platelet response was a part of the hematologic and clinical improvement. In the cases presented the regeneration of platelets and other formed elements of the peripheral blood followed a rather set pattern not mentioned in previous publications.

Experimental. The following folic acid antagonists were used: 4-amino, pteroyl glutamic acid (aminopterin); 4-amino, N¹⁰ methyl pteroyl glutamic acid (a-methopterin); 4-amino, pteroyl aspartic acid (aminofol); and 4-amino, 9 methyl pteroyl glutamic acid (a-ninopterin). These antagonists were administered intramuscularly or orally in approximately the same dosages as recommended by Farber(1,2) and Dameshek(3). Platelet levels were determined by the Rees-Ecker method, normal values in our laboratory being 225,000 to 325,000 per cu mm. The hemoglobin, red cell levels, leukocyte counts and differential counts were also frequently done in addition to bone marrow biopsies by the aspiration method. The leukocytes were studied qualitatively by the supravital method using Janus green and neutral red.

Observations. A definite response, either

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service. The folic acid antagonists were furnished through the courtesy of the Lederle Laboratories Division of the American Cyanamid Company.

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2. Farber, S., *Blood, J. Hemat.*, 1949, v4, 160.

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6. Stickney, J. M., Mills, S. D., Hagedorn, A. B., and Cooper, T., *Proc. Staff Meet., Mayo Clinic*, 1949, v24, 525.

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TABLE I.
Observations on Platelet Response in 9 Patients with Acute Lymphatic Leukemia Treated with Folic Acid Antagonists.

Age, yrs	Sex	Folic acid antagonist	Remission duration, days	Initial platelet count, per mm ³	Platelets, days of therapy to 100,000 per mm ³	Highest platelet level, per mm ³
3	F	aminopterin	110	× 1,000 28	8	× 1,000 310
10	M	aminopterin	240	40	20	295
6	F	aminopterin	200	26	16	264
5	F	amino-an-fol	30	28	28	548
3	M	aminopterin	35	43	27	287
4	F	aminopterin	30	34	29	197
4	F	a-methopterin	150	42	24	394
5	F	aminopterin	30	70	31	360
2	M	a-methopterin				
		aminopterin	55	74	21	310

hematologically and/or clinically, was observed in 25 of the 48 patients with acute leukemia and treated with folic acid antagonists. Eleven patients had an excellent remission both hematologically and clinically and in 14 subjects a definite improvement was noted but leukemic cells could be found in the peripheral blood, and some degree of enlargement of the lymph nodes and spleen could be detected. In the group of 25 patients who responded to some type of folic acid antagonist there were 9 patients who had an initial platelet count under 100,000 per cu mm and 8 of these had a definite rise to normal values and 1 to almost normal levels during their period of remission. All 9 patients were 10 years of age or under and had acute lymphatic leukemia. The accompanying table shows the data in the 9 patients observed. The increased platelet level in most instances was the first evidence of a good hematologic response and usually occurred in third and fourth weeks of therapy. The polymorphonuclear neutrophilic leukocytes began to respond at about the same time or shortly thereafter, this being followed later by a rise in the erythrocyte count and hemoglobin. Enlarged spleens, livers and lymph nodes receded in size. Bone marrow studies revealed a decrease in leukemic cells with a return of normal marrow elements including the megakaryocytes.

It is of interest that in those patients in a state of marked hemorrhagic diathesis the

tendency to bleed disappeared before the platelets became elevated. These patients were given transfusions and hesperidin methyl chalcone, which may have had some effect on the bleeding phenomenon. However, the hemorrhagic tendency in patients not responding to therapy with folic acid antagonists was most difficult or impossible to control.

Discussion. The exact nature of the action of the folic acid antagonists is unknown. Theoretically one would expect a folic acid deficiency to possibly produce a thrombocytopenia, a marked decrease in platelets being observed in the macrocytic hyperchromic type of nutritional anemia. Farber(1) has emphasized the toxic nature of the antagonists. The lesions produced by the toxic effect of the compounds are similar to those produced by a folic acid deficiency in the rat and monkey. Stomatitis, ulceration of the mucous membranes of the mouth, smooth tongue, pharyngitis, and atrophic changes in the intestinal tract have been noted in these folic acid deficient animals. Farber(2) and Dameshek(3) both emphasize the difficulty in determining whether or not the lesions produced by the folic acid antagonists are primarily due to a folic acid deficiency. Our observations have confirmed these statements. Various types of therapy such as vitamin B preparations, including vitamin B₁₂, liver extracts, both crude and concentrated, and folic acid have not been more beneficial than merely

stopping the administration of the folic acid antagonist. It is indeed remarkable that a normal peripheral hematologic picture, including a platelet response, occurs under such therapy. Macrocytic erythrocytes and anisocytosis have been observed(8). A definite explanation of the response cannot be made at present.

Conclusions. In 48 human subjects with acute leukemia treated with folic acid antagonists, there were 9 of the 25 subjects who

responded to therapy who had an initial thrombocytopenia. In 8 cases of acute leukemia there was a return of the blood platelets to normal levels and the megakaryocytes became more numerous in the bone marrow. In one other patient the platelets became elevated to slightly less than normal. The rise in platelets was usually followed by an increase in neutrophilic leukocytes. The regeneration of the red cells and hemoglobin then gradually responded. The periods of remission varied from 21 to 240 days.

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Comparative Tissue Explant Requirements of Columbia-SK, C(M) and M.M. Viruses, and of Theiler's Encephalomyelitis Virus.* (17766)

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Cultivation of the "murine strain of SK poliomyelitis virus"(1,2), here designated Columbia-SK virus(3), has been reported by Sanders and Jungeblut(2). This virus was originally reported as a rodent adapted human strain of poliomyelitis virus (Yale-SK strain), but has recently been established to be a member of the encephalomyocarditis (EMC) group of viruses and to be antigenically unrelated to the original or Yale-SK strain of poliomyelitis virus(4-6). Cultivation of the M.M. virus(7), also a member of the EMC

group(4-6), in tissue explants, has been reported by Jungeblut(8). Enright and Schultz(9) first cultivated the Col. SK, M.M. and C (M) viruses in developing eggs. The last named virus stemmed from passage material of the Lansing strain of poliomyelitis virus and was later established by Schultz and White(6) to be antigenically similar to the Col. SK virus. It has been observed to induce typical flaccid paralysis in occasional rhesus monkeys(9) and to be more virulent for cynomolgus monkeys(6). Cultivation of the GD VII strain of Theiler's encephalomyelitis virus in tissue explants has been reported by Parker and Hollender(10), and its cultivation in eggs by Dunham and Parker(11), and Enright and Schultz(9). Cultivation of the FA strain in eggs has been reported by

* Aided by the Howard Frost Poliomyelitis Research Fund.

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4. Dick, G. W. A., *J. Immunol.*, 1949, v62, 375.

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TABLE I. Results Obtained from Columbia SK, C(M), and M.M. Viruses, and the GD VII and FA Strains of Theiler's Encephalomyelitis Virus, When Grown on Embryo Tissue Explants of Different Kinds. The dilutions given represent ID₅₀ titers; zeros indicate absence of infectivity in 10-1 dilution of the culture fluid in the passages indicated.

Virus	Culture passage	Kinds of explants from mouse embryos										Mincd 5-day chick embryo
		Brain	Lung	Small intestines	Heart muscle	Tail	Tongue	Skeletal muscle	Liver	Kidney	Spleen	
Col. SK	5	10-5	10-4	10-5	10-4	10-4	0	0	0	0	0	10-5
	10	"	"	"	"	10-2	"	"	"	"	"	"
	15	"	10-5	"	"	"	"	"	"	"	"	"
C(M)	5	10-8	10-7	10-7	10-5	10-5	0	0	0	0	0	10-6
	10	"	"	"	"	10-4	"	"	"	"	"	"
	15	"	10-6	"	10-6	10-5	"	"	"	"	"	"
M.M.	5	10-7	10-4	10-4	10-6	10-6	0	0	0	0	0	10-3
	10	"	"	"	10-5	10-5	"	"	"	"	"	"
	15	10-8	"	"	"	10-4	"	"	"	"	"	"
GD VII	5	10-5	10-1	10-1	10-3	10-5	0	0	0	0	0	10-4
	10	"	0	0	10-1	10-1	"	"	"	"	"	"
	15	10-3	"	"	0	10-2	"	"	"	"	"	"
FA	5	10-3	10-2	0	10-2	0	0	0	0	0	0	10-2
	10	"	"	"	10-1	"	"	"	"	"	"	"
	15	10-2	0	"	0	"	"	"	"	"	"	"

Gard(12), and Riordan and Sâ-Fleitas(13).

The purpose of this investigation was to compare the tissue explant requirements of the above viruses in fluid or suspended cell cultures set up with Simms' solution and ox serum ultrafiltrate as described below.

Materials and methods. The Col. SK and M.M. viruses were obtained from Dr. Claus W. Jungeblut in 1942 and 1946 respectively. The source of the C(M) virus has already been stated. The GD VII strain of Theiler's virus was obtained from Dr. Harold H. Pearson in 1945 and the FA strain from Dr. W. McD. Hammon in 1947. The brains and spinal cords of infected mice were harvested soon after the onset of paralysis. These were pooled, ground to a paste in a mortar and made up to a 10% suspension by weight in 50% Martin's peptone solution (MPS II) (14). This suspension was stored overnight in a refrigerator at 4°C, diluted with MPS II to make a 1% suspension, centrifuged at 2,000 rpm for 15 minutes and filtered through a Chamberland L₃ candle. The ID₅₀ of these filtrates and of culture transfers, was determined in the usual manner by inoculating individual 10-fold dilutions, made up with MPS II, into 5 or 6 three- to four-week-old mice, 0.025 cc of each dilution being inoculated intracranially. The ID₅₀ titers of these filtrates ranged from 10⁻⁵ to 10⁻⁷.

The tissue explants employed were obtained from 14- to 16-day-old mouse embryos, the pregnant animals being killed by ether anesthesia. Explants of the following tissues and organs were employed: Brain, lung, small intestine, heart muscle, skeletal muscle (from the thigh), tail, tongue, liver, kidney and spleen. These tissues were placed in separate Petri dishes containing glucosol(15) previously warmed to 37°C. Cultures were also set

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14. Levine, M., and Schoenlein, H. W., A Compilation of Culture Media for the Cultivation of Microorganisms, Baltimore, Williams and Wilkins Co., 1930, p. 308 (medium No. 998).

15. Parker, R. C., *Methods of Tissue Culture*. New York. Paul H. Hoeber, 1938, p. 36.

up with 5-day-old chick embryo tissue, in which the whole embryo was minced and portions added to the nutrient fluid. The individual tissues employed as explants were minced into approximately one mm pieces with curved scissors. One ounce screw top bottles were used as containers. The fluid medium consisted of 3 cc of Simms' (16) solution and 1.25 cc of ox serum ultrafiltrate (16), the choice of these proportions being based on the results of preliminary tests carried out with Col. SK in the presence of brain explants. To this medium 0.25 cc of minced tissue in glucosol solution, and 0.5 cc of an undiluted Chamberland L₃ filtrate of the virus suspension, were added. The cultures were incubated at 37°C, in an incubator provided with a nearly horizontal slowly rotating plate to improve surface aeration of the cultures. Transfers were made at 3-day intervals. In doing this the cultures were centrifuged for 15 minutes at 2,000 rpm, and 0.5 cc of the supernatant was added to the new medium. At the end of 5 or more serial passages the individual cultures were assayed for virus content. This was done on the fluid portion of the medium only, after the cultures had been centrifuged for 15 minutes at 2,000 rpm to eliminate all or most of the tissue particles. The supernatant was diluted to 10⁻¹ with MPS II and this dilution was first tested for viral activity. When this dilution proved active, further dilutions with MPS II were made and tested, the original material being stored in the meantime at -30°C. Culture series which yielded negative results on given explants by the 5th passage were repeated at least once. Virus neutralization tests were carried out at the end of these studies to verify the identity of viruses cultivated.

Observations. The results obtained on different explants with the 5 viruses studied are summarized in Table I.

It will be noted that the Col. SK, C(M) and M.M. viruses grew on explants of certain tissues but not on others, and that the GD VII and FA strains of Theiler's virus were the more exacting in their growth requirements.

It will be noted, moreover, that certain differences in explant requirements were observed with reference to the latter 2 strains. The GD VII strain, but not the FA strain, grew on explants of tail tissue.

Inasmuch as liver and spleen failed to support growth of any of these viruses an attempt was made to determine whether these tissues might contain some extractable inhibiting substance. Cultures of Col. SK were therefore set up with embryo mouse brain tissue, but in which an extract of liver or of spleen in Simms' solution was employed in place of regular Simms' solution. The virus grew as well on this medium as on the regularly prepared medium. In another experiment, an extract of embryo mouse brain in Simms' solution was added to cultures of Col. SK, set up with explants of liver or spleen tissue. Explants of these 2 tissues still failed to yield growth of the virus.

Discussion. The observation of special interest, of course, is the well defined selection of explants exhibited by the first 3 viruses (Col. SK, C(M) and M.M.). While certain quantitative differences were observed, some of which may rest on experimental factors, the most striking feature of the results obtained relates to qualitative differences. All 5 of the viruses might be expected to grow on explants of brain, and possibly also on extra-nervous tissue, but it is not apparent why growth should occur on certain extra-nervous tissues and not on others. A well defined difference exists, for example between explants from heart muscle and skeletal muscle, and between lung and liver as supports of growth for the Col. SK and the two related viruses. Inasmuch as skeletal muscle failed to support growth, it is in a measure understandable why tongue also failed to do so, but not clear why tail tissue supported growth and tongue did not. Since explants of skin alone were not included in these studies, it is not possible to say what role epidermis may have played in the multiplication of virus in explants of tail tissue. Schultz and White (6) have observed that adult mice can be easily infected by members of the EMC group of viruses merely by dipping the clipped tip of the tail into a virus suspension, and have ob-

16. Simms, H., and Sanders, M., *Arch. Path.*, 1942, v33, 619.

served a well defined increase in virus locally, indicating that in adult mice also some component(s) of the tail supports the growth of these viruses.

It has been pointed out that the FA strain of Theiler's virus differed from the GD VII strain in its failure to grow on tail tissue. It also showed a somewhat lower ID₅₀ titer throughout. Both of these viruses, however, require further study from certain standpoints, such as the optimum composition of the fluid medium, length of incubation, etc., before the influence of individual tissue explants can be fully evaluated.

Summary. In a fluid medium consisting of 3 cc of Simms' solution and 1.25 cc of ox-serum ultrafiltrate plus 0.25 cc of selected minced tissue in glucosol solution, the Col. SK, C(M) and M.M. viruses were found to grow freely on tissue explants of brain, lung, small intestine, heart muscle, and tail from

embryo mice, but not on explants of tongue skeletal muscle, kidney, liver, or spleen from the same embryos. Growth of all 3 viruses was also obtained on explants of minced 5-day chick embryos, but not to an equal degree.

The GD VII strain of Theiler's encephalomyelitis virus was carried through 15 culture passages on explants of brain and tail tissue, but failed to show well defined or continued growth on explants of other tissues and organs from embryo mice. Unlike the GD VII strain, the FA strain of Theiler's virus failed to grow on explants of tail tissue. Both strains grew on explants of chick embryo tissue, but the FA strain grew less freely than the GD VII strain, even on explants of mouse brain tissue, under the conditions described.

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Comparative Specific Gravities of Normal and Tumor Tissues.* (17767)

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Recently we observed that increased pressures were capable of inhibiting the growth of Sarcoma 180 in mice(1). This finding indicated the possibility that tumor tissue may have a different density from normal tissue. Preliminary studies on flotation of Sarcoma 180 in sodium chloride solutions appeared to support this possibility. Fawcett, Vallee, and Soule(2) have recently shown that blood cells sediment at different rates than cancer and mesothelial cells in albumin solutions.

In view of these findings, it was decided to investigate more systematically the specific gravity of various mouse, rat and human tu-

mors.

Materials and methods. The transplantable mouse tumors used in these experiments were Crocker sarcoma 180 Lewis sarcoma T241, sarcoma MA387, Patti myxosarcoma, mammary adenocarcinoma EO 771, Wagner osteogenic sarcoma, Ridgway osteogenic sarcoma, Patterson lymphosarcoma, Mecca lymphosarcoma, Harding-Passey melanoma and Andervont hepatoma. The original myxosarcoma was induced by methylcholanthrene, while the hepatoma was induced by *o*-aminoazotoluene. Sarcoma 180, myxosarcoma and the melanoma were grown in albino mice of the Rockland Farms strain; osteogenic sarcoma, lymphosarcoma and sarcoma MA 387 were grown in mice of the AKm strain (leukemic); sarcoma T241 and adenocarcinoma EO 771 were grown in C57 black mice in which they originated, and hepatoma was grown in strain C mice. The transplantable

* This work was supported by a grant made to the Sloan-Kettering Institute for Cancer Research by the American Cancer Society.

1. Arkin, A. M., and Sugiura, K., *Cancer Research*, in press.

2. Fawcett, D. W., Vallee, B. L., and Soule, M. H., *Science*, 1950, v111, 34.

rat tumors used in the present study were Flexner-Jobling carcinoma, Jensen sarcoma, Sarcoma R39 and Walker carcino-sarcoma 256. These tumors were grown in albino rats of the Sherman strain. Subcutaneous implantations of tumors (small pieces of tumor, each measuring about 1.5 mm³ and weighing approximately 6 mg) into healthy young animals (18-22 g mice; 100-125 g rats) were carried out by the usual trocar method (a single implant into the right axillary region).

The animals were given food (Purina Laboratory Chow) and water *ad libitum*. In our earliest observations on this phenomenon a very simple experiment was done. Sarcoma 180 was implanted into mice of the Rockland Farms albino strain in the usual axillary region. One week later the animal was killed with ether and pieces of tumor, liver, kidney, spleen, heart and skeletal muscle were dropped into a beaker of saline solution. The specific gravity of the solution was adjusted by adding salt or water, as necessary, until the tumor just floated. In such a solution, the

other tissues mentioned above[†] invariably sank to the bottom (Fig. 1). This behavior has been constant in several different batches of such tumorous mice tested at intervals during the last sixteen months.

More recently, the following technic was adopted for determining the specific gravity of tumor and normal tissues. After killing the tumor-bearing animal with ether anesthesia, the tumor was removed, and adherent fat and fascia cut away. A piece of tumor tissue 5 to 10 mm in diameter was cut from a healthy, non-necrotic area at the periphery of the tumor, quickly rinsed in 0.85% sodium chloride solution in water, and then touched to filter paper to remove the excess saline. It was then immediately dropped into a beaker of sodium chloride solution of known specific gravity (solutions of each percentage from 6% to 13% NaCl[‡] were available). The range of specific gravity was estimated by determining the density of solution in which the tumor would sink and in which it would float. A similar procedure was repeated with spleen, kidney, liver, heart, and thigh muscle of the host. All determinations were done within 10 to 30 seconds since the observations became unreliable after longer immersion in salt solution, the tissues becoming heavier, probably by absorption of salt.

At the time the specific gravity determinations were made, the tumor-bearing mice were about 2 to 4 months old and weighed about 20 to 35 g. The tumor-bearing rats were about 2 to 4 months old and weighed about 150 to 200 g. Sarcoma 180 was about 7 days after implantation; sarcoma T241, Ridgway osteogenic sarcoma, and both lymphosarcomas were about 2 weeks old; Wagner osteogenic sarcoma was about 3 weeks old; melanoma was about 6 weeks old; and hepatoma was about 10 weeks old. It is to be noted that these respective periods are the best age for tumor transplantation.

Results. The specific gravity of normal mouse tissues of various strains lies between

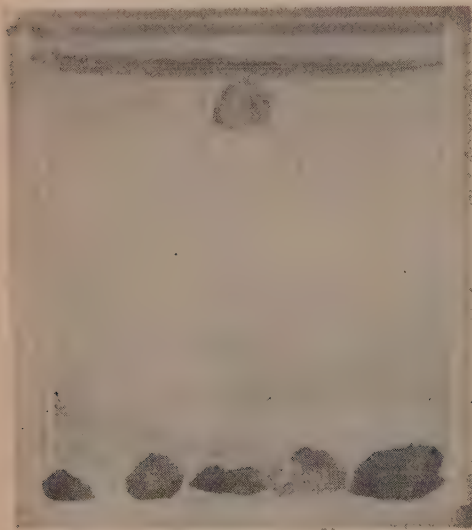


FIG. 1.

Sarcoma 180 floats in 8% sodium chloride solution. Heart, kidney, spleen, muscle and liver sink. (All tissues from the same mouse).

[†] Testis was even lighter than tumor, and would float in solutions in which Sarcoma 180 sank.

[‡] The specific gravities of 6%, 8%, 10%, and 12% NaCl solutions at 20°/4°C. are 1.0413, 1.0559, 1.0707, and 1.0857, respectively.

TABLE I.
Flotation of Normal Mouse Tissues of Various Strains (% NaCl at 22°C).

Tissue	Rockland Albino		C57 Black		C ₃ H		AKm		C	
	S*	F*	S	F	S	F	S	F	S	F
Heart	9	10	9	10	9	10	9	10	9	10
Liver	11	12	11	12	11	12	11	12	11	12
Spleen	9	10	10	11	10	11	10	11	10	11
Kidney	9	10	9	10	9	10	9	10	10	11
Muscle	9	10	10	11	9	10	9	10	10	11

S = sink.

F = float.

the limits summarized in Table I. In all 3 tables, each determination summarizes determinations on 2 to 6 animals, which were practically uniform in every case. Heart and kidney were lightest, liver the heaviest tissue, in every animal. Although there are minor variations in the various strains of mouse, the pattern for each strain was quite constant. Specific gravities of normal tissues of male and female animals were essentially the same.

The specific gravities of certain transplantable mouse tumors are given in Table II. It will be noted that the tumor is always markedly lighter than any other tissues tested except in the case of the Harding-Passey melanoma and the Andervont hepatoma. Both of these tumors had specific gravities equivalent to that of a solution of 9 to 10% sodium chloride—comparable to some of the normal organ tissues. However, when these tumors and the other organs of the host were placed in 9% saline, and dry sodium chloride added slowly, with stirring, the tumors would float before the

TABLE II.
Flotation of Transplantable Mouse Tumors.

Tumor	Conc. of NaCl sol. at 22°C (%)				
	6	7	8	9	10
Crocker sarcoma 180	S*	F*			
Lewis " T241	S	F			
Sarcoma MA387	S	F			
Patti myxosarcoma	S	F			
Mammary adenocarcinoma EO 771	S	F			
Wagner osteogenic sarcoma	S	F			
Ridgway " "	S	F			
Patterson lymphosarcoma	S	F			
Mecca " "	S	F			
Harding-Passey melanoma				S	F
Andervont hepatoma				S	F

* S indicates sink; F indicates float.

TABLE III.
Flotation of Normal and Tumor Tissues (Rat).

Tissue	Conc. of NaCl sol. at 22°C (%)					
	6	7	8	9	10	11
Flexner-Jobling carcinoma	S*	F*				
Walker carcinoma	S	F				
carcinoma 256						
Jensen sarcoma		S	F			
Sarcoma R39		S	F			
Heart			S	F		
Liver					S	F
Spleen				S	F	
Kidney			S	F		
Muscle					S	F

* S indicates sink; F indicates float.

other tissues (cf. Sarcoma 180 in Fig. 1). Hence even these relatively heavy tumors were lighter than normal organs in the same mouse. Moreover, the specific gravity of hepatoma, though relatively high, is much less than that of normal liver tissue.

Similar studies in the rat are summarized in Table III. The same observations hold true in this animal, all of the tumors being of lesser density than any of the other organs tested. All determinations of tumors were done on normal-appearing tumor from the periphery, both in the rats and in the mice. However, in addition to normal tumor, areas of Flexner-Jobling carcinoma which were at least 90% necrotic were tested, and yielded the same readings as healthy tumor. Ridgway osteogenic sarcoma in AKm mice yielded a reading of 6 to 7 whether healthy tumor, 50% necrotic areas, or hemorrhagic areas were tested.

Determinations of specific gravity of two types of spontaneous tumors in mice yielded similar information. Spontaneous mammary adenocarcinoma in Rockland albino mice had

a density equivalent to 6 to 7% sodium chloride solution. Spontaneous mammary adenocarcinoma in C₃H mice read 7 to 8%.

In a limited experience with human material, the results seem similar to those in the experimental animal. A single specimen of carcinoma of the rectum obtained at operation read between 7 and 8. A retroperitoneal lipomyxosarcoma removed at autopsy floated in 3.5% sodium chloride solution (this was a grossly fatty tumor). In this case, and 3 cases of lymphosarcoma and one of myeloblastic leukemia examined postmortem, heart and muscle ranged from 7 to 8 or 8 to 9; kidney, 7 to 8 (6 to 7 in lipo-myxosarcoma and myelogenous leukemia); spleen, 8 to 9; liver, 9 to 10; lymphosarcoma read between 6 and 7.

The organs of mice bearing Patterson lymphosarcoma (2 weeks after tumor implantation) were lighter than those of normal AKm mice (heart 8-9; liver 10-11; spleen 8-9; kidney 8-9; muscle 9-10). The liver and spleen were grossly enlarged and infiltrated with tumor in these mice.

Summary. 1. The specific gravity of tumor and normal tissues of mice and rats was determined by flotation in sodium chloride solutions of known concentrations.

2. The density of tumor tissue was always less than that of heart, liver, spleen, kidney, and muscle of the host.

3. Several human tumors were tested and exhibited the same property.

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Effect of Infectious Myxoma Virus on Glycolysis of Chorioallantoic Membrane of Chick Embryo.* (17768)

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Little is known of the effect of viruses on the cell metabolism of the infected host. Most of the information originates from experiments on bacteriophage and from studies of the behavior of viruses in tissue culture. The chorioallantoic membrane of the developing chick embryo appeared to be a suitable model tissue for the study of cell metabolism in the presence of a virus for the following reasons: a) It can be infected readily with a great variety of viruses, and the membrane can be used conveniently for *in vitro* metabolic measurements. b) The development of chemical and morphological changes can be followed at any desired time intervals, thus permitting chronological correlations hitherto difficult to obtain on mammalian tissues.

The present paper summarizes some obser-

vations made on the glycolysis of chorioallantoic membranes of 11-day-old chick embryos which had been inoculated with myxoma virus. The measurement of respiration and glycolysis was carried out 48 hours after inoculation. The experiments reported here exemplify the usefulness of this technic (see point a), while time relationships between chemical and morphological effects (point b) will be reported elsewhere(1). Infectious myxoma virus as an infecting agent appeared particularly suitable for these studies, since this virus causes characteristic lesions on the membrane without killing the embryo itself(2-5). Moreover, the

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2. Lush, D., *Austral. J. Exp. Biol. and Med. Sci.*, 1937, v15, 131.

3. Hoffstadt, R. E., and Pilcher, K. S., *J. Bact.*, 1938, v35, 353.

4. Hoffstadt, R. E., and Pilcher, K. S., *J. Bact.*, 1939, v36, 286.

5. Haagen, E., and Dscheng-Hsing Du, *Zbl. Bact. I., Orig.*, 1938, v143, 23.

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† With the assistance of Miss H. E. Sherrard.

behavior of this tumor-producing virus is of intrinsic interest in relation to some aspects of tumor development.

Experimental. In the present experiments the inoculum consisted of a suspension of chorioallantoic membranes infected with myxoma virus which had undergone 39 consecutive passages in the embryonated egg. The infected membranes were ground in a Pyrex grinder to a 10% suspension in 0.85% NaCl. This suspension was divided in ampoules each containing 1 ml of the material and then lyophilized. By this method a uniform infective dose could be secured in successive experiments. Just before use the contents of the ampoules were reconstituted with 1 ml of sterile distilled water and diluted ten-fold with 0.85% NaCl. A sample of 0.3 ml of this final dilution (10^{-2} concentration by weight containing approximately 100 egg infective doses of the virus) was dropped on the chorioallantoic membrane, the egg rotated to insure even virus distribution, then incubated at 35°C for 48 hours. Control eggs were inoculated with a similarly prepared suspension of lyophilized normal chorioallantoic membrane or with distilled water. After incubation the eggs were opened and the central part of the membrane cut out and immediately dropped into ice cold Krebs-Henseleit solution(6). The membranes were washed in this solution several times, then blotted dry on a filter paper, cut into 3-4 mm long strips, weighed on a torsion balance, and transferred to ice chilled Warburg respirometer flasks. The wet weight of the membranes per flask varied between 90-125 mg. Each respirometer flask contained Krebs-Henseleit solution (with 10 mg/ml glucose) and 0.2 ml 20% KOH in the center well. The volume was made up to 3 ml with Krebs-Henseleit solution, the flasks were gassed with 100% O₂ for 5 minutes, and O₂ consumption measured for 60 minutes at 37°C. The values of O₂ consumption were calculated in terms of QO₂ from the amount of O₂ absorbed in one hour. The aerobic lactic acid formation was also determined. Lactic acid analyses were car-

ried out according to Barker and Summerson(7). In each experiment the dry weight of the membranes was measured and found to vary between 4.8 and 6.9%. The conversion of hexose diphosphate to 3-phosphoglyceric acid was determined manometrically according to Kennedy and Lehninger(8). Zymohexase activity was measured by reading the optical density of the dinitrophenylhydrazine chromogen in the spectrophotometer at 540 m μ using the test system of Sibley and Lehninger(9). For these enzyme assays 0.9% KCl homogenates or centrifuged (6,300 x g for 15 minutes) extracts were used. Since the mechanism of glucose fermentation and oxidation of the chorioallantoic membrane was hitherto unknown, an aerobic fermentation experiment was carried out on a larger scale. One gram each of normal and infected membranes was incubated with 500 mg glucose in 10 ml Krebs-Henseleit solution (containing 20 mg P in the form of KH₂PO₄ and MgSO₄ in final conc. 0.005 M) for 5 hours in 100% O₂ atmosphere at 36.8° C. The reaction was stopped with 10 ml cold 10% trichloroacetic acid, and the membranes were then homogenized and extracted. Extraction was carried out 3 times with 5% trichloroacetic acid, the final volume made up to 50 ml. The esterification of inorganic P, the labile P (Δ_7 P) of the Ba insoluble fraction, and the alkali labile P of the alcohol precipitable fraction were determined as outlined by Umbreit *et al.*(10). Phosphorus analyses were made according to Gomori(11), keto acids determined according to Friedemann and Haugen(12). Since the dry weight of the membranes did not differ significantly (normal 5.1%, infected 4.8%),

7. Barker, J. B., and Summerson, W. H., *J. Biol. Chem.*, 1949, v138, 535.

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9. Sibley, J. A., and Lehninger, A. L., *J. Biol. Chem.*, 1949, v177, 859.

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11. Gomori, G., *J. Lab. and Clin. Med.*, 1942, v27, 955.

12. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.

6. Krebs, H. A., and Henseleit, K., *Z. Physiol. Chem.*, 1932, v210, 33.

TABLE I.
Effect of Infectious Myxoma Virus on O₂ Consumption and Aerobic Lactic Acid Formation of Chorioallantoic Membrane of the Chick Embryo.

No. of exp.	QO ₂		Lactic acid	
	Normal	Infected	Normal	Infected
1	13.2	14.8	19.2	32.0
	12.4	11.0	14.7	28.6
2	13.8	14.7	18.5	40.1
	15.8	10.8	13.6	38.0
3	14.9	18.2	15.5	48.4
	15.2	17.0	14.0	39.2
4	19.7	16.6	15.8	50.0
	18.1	21.0	16.8	49.0
m	15.3	15.5	16.0	40.6
δ	2.6	3.66	2.1	7.35
ε	±0.92	±1.28	±0.74	±1.92
d	0.24		12	

m = mean value.

δ = difference from mean (±).

n = number of measurements.

QO₂ = microliters of O₂ per mg dry wt per hr.

Lactic acid = micrograms of lactic acid per mg dry wt per hr.

$$\delta = \frac{\sqrt{\sum d^2}}{\sqrt{n-1}} \quad \epsilon = \frac{\delta}{\sqrt{n}} \quad d = \frac{m_1 - m_2}{\sqrt{\epsilon^2 + \epsilon^2}}$$

the results of the incubation experiments were expressed in terms of 1 g of fresh weight.

Results and discussion. The oxygen consumption and aerobic lactic acid formation of normal and infected membranes are summarized in Table I. The metabolism of 2 separate samples of each membrane was compared in 4 series of experiments. Simple statistical evaluation of the data revealed an increased aerobic lactate formation from glucose by the infected membranes as compared to the normals. The metabolism of control membranes, i.e., those inoculated with 0.3 ml of suspension of lyophilized chorioallantoic membrane containing no virus, was identical with the normal membranes. The marked increase in glycolysis exhibited by the infected membranes, as contrasted with these controls, indicates that this metabolic effect is due to the presence of the virus.

The question arises as to whether or not the virus itself has glycolytic enzyme activity. This appears unlikely, based on the observation that the virus titer and increase in gly-

colysis do not correspond, and the increase in the rate of glycolysis persists, even increases, after the virus titer has decreased to a lower level. This finding will be reported elsewhere(1). The mechanism of increased glycolysis was further demonstrated by comparing the rate of conversion of the Harden-Young ester to 3-phosphoglyceric acid by homogenates of normal and infected membranes. (See graph.) Since the rate of fermentation of Harden-Young ester to triose phosphate was greater in the infected membranes, the conclusion seems warranted that the activation of a glycolytic enzyme occurred in the chorioallantoic membranes inoculated with virus. It was found, furthermore, that in 0.9% KCl extracts of infected membranes the zymohexase activity was markedly increased over the enzyme activity of the extract of normal membranes. The magnitude of the zymohexase activation is shown by the following typical experiment. The optical density of the triose chromogen (at 540 mμ) formed during 15 minutes' incubation at 37°C was 0.290 for an

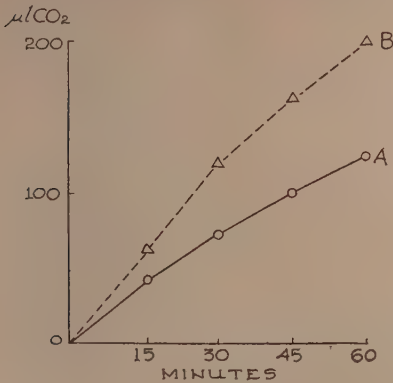
In Vitro formation of 3-phosphoglyceric acid

FIG. 1.

In vitro formation of 3-phosphoglyceric acid from hexosediphosphate by 0.9% KCl homogenates of normal (A) and myxoma-infected chorioallantoic membranes of the chick embryo (B). Each flask contained: Tissue homogenate (corresponding to 200 mg membrane); 0.03 M HDP; 0.002 M Na_2HAsO_4 ; 0.02 M NaF; 0.02 M Pyruvate; 0.02 M Nicotinamide; 0.048 M Na HCO_3 . Reaction started after 15 minutes incubation by the addition of 100 γ DPN from the side arm. Total volume of reactants 3 ml. Gas = 95% N_2 + 5% CO_2 . Temperature = 37°C.

extract made from 20 mg of normal chorioallantoic membrane in contrast to 0.480 in the case of an equivalent extract of infected membrane. Further data on zymohexase activation will be presented elsewhere. These measurements were corroborated by the analytical results obtained from the fermentation experiment, using 1 g each of pooled normal and of pooled infected membranes. The formation of triose phosphates (determined as alkali labile P of the alcohol precipitable fraction), keto acids (expressed in terms of pyruvic acid), and lactic acid from glucose was

TABLE II.
Formation of Triose Phosphates, Keto- and Lactic Acids During Glucose Fermentation.

Metabolite	Normal membrane, mg	Myxoma infected membrane, mg
Triose phosphates	.017	.033
Keto acids (in terms of pyruvic acid)	.023	.051
Lactic acid	.575	2.025

Results expressed in terms of mg formed by 1 g of membrane in the incubation mixture during 5 hr.

increased in the fermentation mixture containing the infected membranes. Results of this typical experiment are summarized in Table II. The esterification of inorganic P occurred to the same extent in both fermentation experiments. During 5 hours' incubation 5.3 mg of the 20 mg of added inorganic P were esterified, 3.1 mg were taken up in the "phosphocreatine" fraction (difference between inorganic and Ca precipitable P), and 2.2 mg in other fractions. In both experiments 0.7 mg pyrophosphate P (presumably adenylypyrophosphates) were formed (as determined by the 7 minute P of the Ba insoluble fraction). While the pathway of carbohydrate metabolism of the chorioallantoic membrane is at present unknown, our data support the view that glucose is metabolized by way of phosphorylation.

Interestingly enough Racker(13) found that certain neurotropic viruses inhibit brain glycolysis, which raises the question as to whether or not the activation of glycolysis is a specific effect of the myxoma virus. Experiments with other viruses are in progress in this laboratory. Barron(14) found that rabbit myxoma tissue, similar to other neoplastic tissues, has a high aerobic glycolysis. From the experimental evidence presented it appears likely that myxoma virus increases the glycolysis of the chorioallantoic membrane by activation of a glycolytic enzyme (zymohexase). It may be that a similar mechanism is responsible for the increased rate of glycolysis in rabbit myxoma tissue.

Summary. The lactic acid formation from glucose is increased in the chorioallantoic membrane of the chick embryo after inoculation with infectious myxoma virus. This increase must be due to an activation of glycolysis since respiration at this stage is not impaired.

The authors are indebted to Dr. Lewis Thomas for his encouragement and helpful criticism offered in the course of these experiments.

13. Racker, E., and Krinsky, I., *J. Exp. Med.*, 1947, v85, 715.

14. Barron, E. S. G., *J. Exp. Med.*, 1932, v55, 829.

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Effect of Digitoxin on Latent Period of Frog Gastrocnemius Muscle. (17769)

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The accepted action of digitalis on heart muscle according to Sollman(1) is the production of a "lowered threshold of irritability, more powerful and more prolonged contraction with corresponding lengthening of the refractory period." Early workers, Bradenburg(2) and Gies(3) working with isolated frog heart preparations demonstrated this prolongation as seen by an increased duration of contraction period. Little work has been done recently on this particular phase of digitalis action. The present paper is a report of the action of digitoxin on the latent period of isolated frog gastrocnemius muscle. In these experiments the latent period may be defined as "the period of apparent inactivity between the application of an effective stimulus and the earliest evidence of tissue response(4)." It is not to be confused with the electrocardiographic P-R interval which represents the period between electrical depolarization of auricle and that of ventricle.

Materials and methods. Frog gastrocnemius muscles were mounted immediately after isolation to a myograph consisting of a fixed point and writing lever connected with a suitable tissue bath for immersing the muscle in digitoxin and the control solution of 0.7% sodium chloride. The myograph was mounted to a kymograph modified for high speed work using a tuning fork timer with an automatic drum key for applying break stimuli from a Porter induction coil after the design and method of Guthrie(4,5). By this method latent periods were graphically recorded on a

smoked drum from the muscle contraction obtained by the direct application of maximal stimuli. Duration of the latent period was measured from the time of recorded stimulation to the onset of muscular contraction using the recorded tuning fork vibrations for time intervals. Control latent periods were determined with the preparation constantly immersed in 0.7% sodium chloride solution in 9 experiments. Latent periods were also determined with the muscle immersed in digitoxin solution following a single saline control determination in 12 experiments. Digitoxin* was freshly prepared in 0.7% sodium chloride solution and dilutions of 1:25,000, 1:50,000 and 1:100,000 were used.

Results. Control latent periods have been repeatedly determined on 9 preparations of isolated frog gastrocnemius muscle after immersion in 0.7% sodium chloride solution after being mounted on the myograph for a total time interval of 124.4 minutes. The results which are summarized in Table I demon-

TABLE I.
Control Latent Period (0.7% NaCl).

Frog	Latent period (milliseconds)			
	1	2	3	4
B	10	10	10	10
F	11	11	11	11
K	11	11	11	11
P	9	9	10	9
Q	10	11	11	12
R	11	11	10	12
S	13	13	13	13
T	11	12	12	14
U	11	11	11	—
Mean value	10.7	11	11	11.5
Mean time (min.) after immersion	29.8	63.9	92.4	124.4

5. Guthrie, C. C., Contributions to Practical Physiology and Pharmacology, published by the author, 1915.

* Digitoxin was supplied through the courtesy of Dr. G. E. Farrar, Jr., of Wyeth, Inc.

1. Sollman, T., A Manual of Pharmacology, 7th Ed., W. B. Saunders Co., Philadelphia, 1948, p. 454.

2. Bradenburg, K., *Arch. f. Anat. V. Entwicklungsgesch.*, Leipzig, 1904, 384.

3. Gies, K., *Z. f. Biol.*, 1927, v86, 427.

4. Guthrie, C. C., Laboratory Directions in Experimental Physiology, 2nd Ed., Edwards Bros., Ann Arbor, 1947.

TABLE II.
Latent Period After Digitoxin.

Frog	Control latent period	Strength digitoxin × 1000	Latent periods (milliseconds)						
			1	2	3	4	5	6	7
A	12	1:100	12	12	15				
C	13	"	10	11	14	18			
D	5	"	7	14	14	15			
E	11	"	11	11	11	16	17		
G	10	1:50	11	12	14	13	17		
M	9	"	12	13	13	13	14	17	
N	11	"	12	12					
O	9	"	12	13	13	13	14	17	
H	10	1:25	11	13	14	14	15		
I	11	"	11	11	10	11	12	15	17
J	10	"	10	10	10	12			
L	9	"	10	12	13	12	14		
Mean value	10		10.7	12	12.8	13.7	14.7	16.3	17
Mean time (min.) after immersion	29.8	33.4	59.9	81.7	107.3	126.5	137.2	140.	155.

strate that the latent periods remained constant both when compared with each other and over the total time period. Table II summarizes the results of the determinations of latent periods of 12 similar isolated gastrocnemius preparations in which digitoxin of varying strengths was used as a bath material immediately following a preliminary control latent period determination. Following immersion in the saline bath the preliminary latent period determinations were made after a mean time of 29.8 minutes and the muscle was immersed in digitoxin solution at a mean time of 33.4 minutes. The mean value of the preliminary latent period control of 10.0 milliseconds in this group compares favorably with that of 10.7 milliseconds for the initial

latent period of the saline control series presented in Table I. The prolongation of the latent periods of isolated frog gastrocnemius muscles immersed in digitoxin solution can be seen in Table II in which the mean latent period values progressively increase with time from the first mean value after addition of digitoxin of 10.7 milliseconds to the final 17.0 milliseconds. From this data it is evident that after digitoxin was added the duration of latent period increased while that of the control remained relatively constant.

Summary. Digitoxin solution applied by immersion technic prolongs the latent period of isolated frog gastrocnemius muscle.

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Anatomy of the Thoracic Vagus in the Dog and a Technic for Its Interruption.* (17770)

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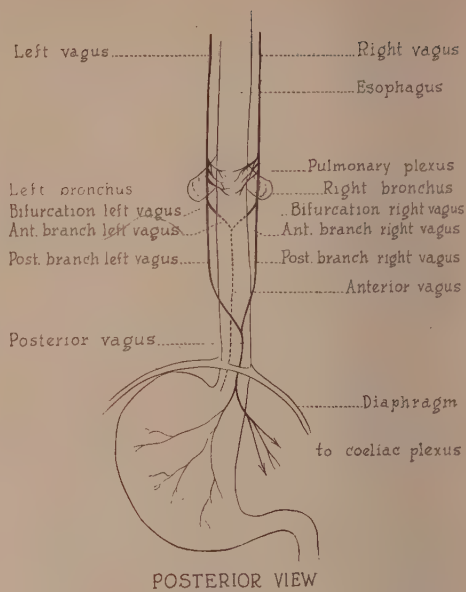
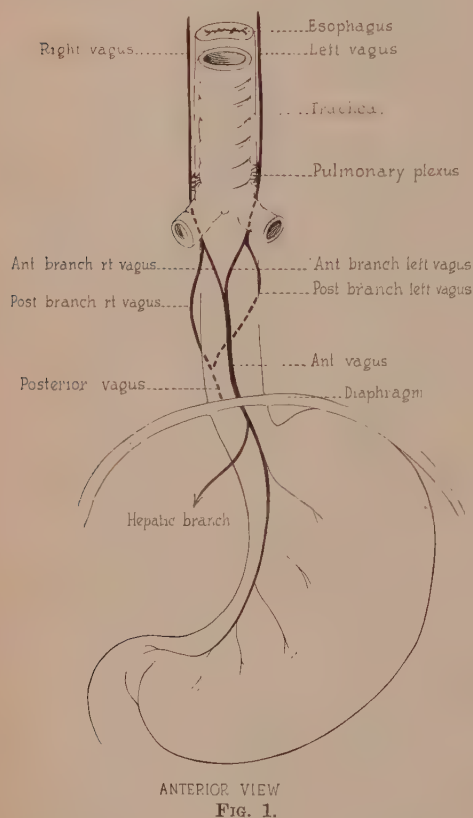
Preparatory to observing the role of the vagus nerve in the genesis of peptic ulcer produced experimentally in dogs by cinchophen(1),

it was mandatory to have a procedure which would free the stomach from vagus nerve supply. It has been established(2) that the

1. Hilsabeck, J. R., and Hill, F. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 155.

2. Hartzell, J. B., *Am. J. Physiol.*, 1929, v91, 161.

stomach of the dog is vagally denervated only when bilateral vagotomy is performed supra-diaphragmatically but we were unable to find an adequate description of the technic involved. In addition, the course of the nerve in the thorax as given by various authors (3-5) was not detailed enough to serve as a basis for such a procedure. Consequently, it was necessary to observe the course of the vagi in a significant number of dogs and, from these observations, to devise an operative procedure, applicable to any dog, which would insure disruption of the thoracic vagi at a level between the hilus of the lung and the diaphragm.



Schema of the thoracic vagi in the dog.

Since there is often a demand for a dog with the vagi disrupted transthoracically below the level of the hilus of the lung but above the diaphragm, it was felt that our findings, as well as a description of the surgical technic evolved, would be of value to experimental workers especially in view of the paucity of such information in the literature.

Anatomical Course of the Vagus Nerve in the Thorax of the Dog. After observing the course of the vagus nerves in the thoraces of 42 dogs coming to autopsy for various reasons, it was apparent, in contrast to man (6,7), that both nerves pursued a constant course (Fig. 1 and 2) deviating from the normal only in diameter of the branches. The typical description, which follows, was taken from the autopsy protocol of one of the animals. In this dog the course of the vagus nerve was easily demonstrated in the thorax and neck. The ribs were cut away, the nerves dissected black India ink applied to the vagi along with the phrenic nerves, and photographs taken to

3. Jemerin, E. E., and Hollander, F., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, v38, 139.

4. McCrear, E. D., *J. Anat.*, 1924, v59, 18.

5. Bradley, O. C., *Topographical Anatomy of the Dog*, Macmillan Company, New York, 1927.

6. Bradley, W. F., Small, J. T., Wilson, J. W., and Walters, W., *J.A.M.A.*, 1947, v133, 459.

7. Miller, E. M., and Davis, C. B., Jr., *J.A.M.A.*, 1947, v133, 461.

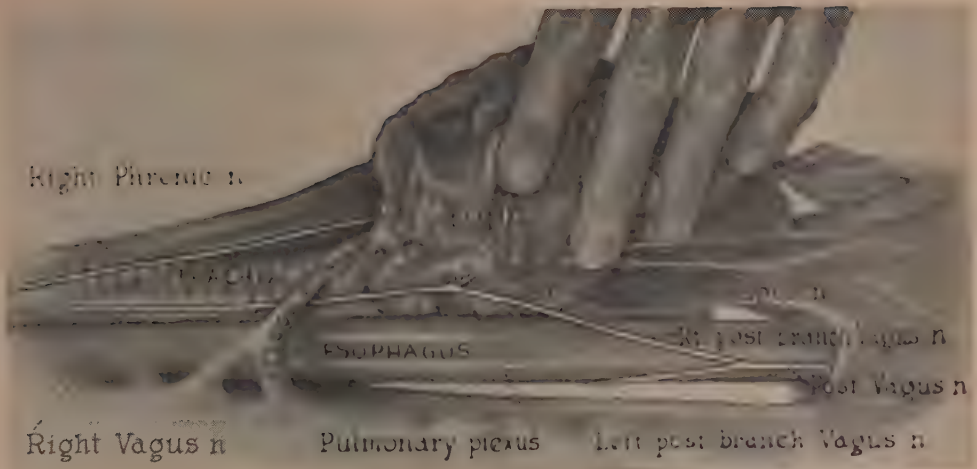


FIG. 3.

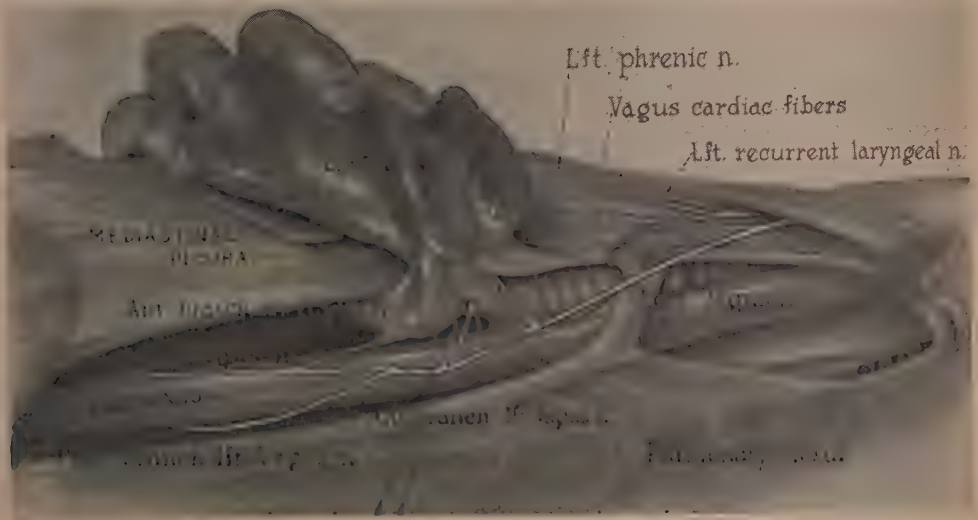


FIG. 4.

Relations of the thoracic vagi in the dog. The mediastinal pleura has not been represented fully in order to insure better visualization.

show the course and relations of the phrenic and vagus nerve on each side. Drawings were then made from the photographs (Fig. 3 and 4).

As the vagus nerves parted company with their respective carotid sheaths, each on entering the thorax gave off showers of nerve fibers, which ran to the base of the heart. Shortly thereafter, each vagus gave off a

recurrent laryngeal nerve, the left as it crossed the aortic arch anteriorly, medial to the left subclavian artery, and the right as it crossed anterior to the brachio-cephalic artery. Then, running in the mediastinal pleura along the lateral walls of the trachea, the right passing under the azygos vein, the left having crossed the ascending aorta diagonally, both vagi passed the hili of the respective lungs pos-

teriorly. As they did so, numerous fibers were given off to each hilus, where the fibers united to form the pulmonary plexus, while the main trunk of the vagus continued caudad.

Below the level of the tracheal bifurcation, the nerves came to lie on the lateral walls of the esophagus, still invested in the mediastinal pleura. Immediately below this level, the right vagus divided into an anterior and a posterior branch. The left vagus divided into an anterior and a posterior branch a little farther caudad. As soon as the anterior branch of the right vagus was given off, it crossed the esophagus anteriorly and united with the corresponding branch of the left vagus in the antero-lateral plane of the esophagus, approximately halfway between the level of the hilus of the lung and the diaphragm. The nerve thus formed, the *anterior vagus*, continued caudad with the esophagus and pierced the diaphragm a little to the left of the anterior plane of the esophagus. The nerve remained covered by, and invested with, mediastinal pleura during its entire progress through the thorax.

The posterior branch of the right vagus continued to run caudad and traversed the length of the esophagus on the right beginning anteriorly and gradually sloping posteriorly. A short distance before piercing the diaphragm, it was joined by the posterior branch of the left vagus to form the *posterior vagus*, which accompanied the esophagus through the diaphragm in the postero-lateral plane. The course of the left posterior vagus was a gradual sloping one *behind* the esophagus, passing almost the entire distance between hilus and diaphragm before coming to lie on the right postero-lateral wall of the esophagus where it joined the posterior branch of the right vagus.

Esophageal fibers were constantly given off by both vagus nerves in their course through the chest.

Comment. The course pursued by the right and left vagus nerves in the thorax as observed at the autopsy table agrees with that described by Jemerin and Hollander(3); however, in contrast to the report of McCreag(4), the right and left vagus nerves divided, below the level of the lung roots, usually into one anterior and one posterior branch. In only 2 dogs it was found that the left vagus divided

into 3 branches, one going anterior and the other 2 coursing posterior. The supernumerary third branch was insignificant in comparison to the other 2; it also joined the right posterior vagus about 5 mm from the junction of the other left posterior vagus with the right. Plexus formation about the esophagus was observed in none.

Technic for Intrathoracic Vagotomy in dogs.
Anesthesia. Intratracheal ether or a combination of morphine-pentobarbital sodium parenterally was used. In our hands, the latter was preferable. The fasting dog is given morphinae sulfas, 3 mg per kilo of body weight, intraperitoneally. This is followed in 30 minutes by 5 mg of pentobarbital sodium per kilo of body weight, intraperitoneally or intravenously. In about 5 minutes anesthesia is complete and a metal cannula, which is part of a positive pressure system, is inserted through the mouth into the trachea, and the jaws closed.

Surgical procedure. Placing the animal on its left side, the right thorax is entered aseptically in the seventh or eighth interspace through a 4 inch incision whose center lies in the mid-axillary line. Care should be taken to avoid the intercostal artery, vein and nerve, superiorly. A self-retaining retractor is inserted between the ribs and spread. At this point the anesthetist begins positive pressure.

Using a wet gauze sponge, the lower lobe of the right lung is grasped gently and pulled superior and anterior. This places the mediastinal pleura on the stretch for it is a continuation of the visceral pleura reflected off the lung. Thus, the mediastinum is exposed from the hilus of the lung to the diaphragm and the right vagus is easily identified running in the mediastinal pleura, posterior to the hilus of the right lung. This is in contrast to the right phrenic nerve, which passes anterior to the hilus, intimate with the pericardium. The mediastinal pleura is then incised with a long-handled knife just anterior to the right vagus about half-way between hilus and diaphragm. As a matter of fact, due to the long chest of the dog and the relatively high dome of the diaphragm, it is difficult to open the pleura at a lower level.

The right vagus is then hooked and a

hemostat applied. Tracing the vagus cephalad by pulling caudad on the hemostat, the division of the right vagus is found slightly inferior to the level of the hilus of the right lung. Consequently, the hemostat is on the posterior branch of the right vagus. By pulling cephalad on the posterior right vagus and following it along the lateral wall of the esophagus, the junction of this branch with the posterior branch of the left vagus is easily located slightly above the diaphragm. Here, the left vagus has just emerged from behind the esophagus. Maintaining cephalad traction on the hemostat, two more hemostats are applied distal to the juncture and approximately $1\frac{1}{2}$ to $3\frac{1}{2}$ cm of the posterior vagus, formed by this union, resected. The cut ends are ligated with cotton.

Keeping in the mediastinum and crossing the esophagus anteriorly, the left vagus is

easily identified running parallel to this structure invested with mediastinal pleura. After hooking the nerve, it is dissected partially free and a hemostat applied. By creating caudad traction on the hemostat, the junction of the anterior branches of the vagus nerves is brought into view. This union is below the bifurcation of the left vagus. The hemostat, therefore, is on the *anterior vagus*. Another hemostat is applied and $1\frac{1}{2}$ to $3\frac{1}{2}$ cm or more of the nerve removed, the cut ends also being ligated.

Thus, both vagus nerves have been disrupted in the thorax and the operation is complete except for wound closure.

Summary. 1. The thoracic vagus as seen in 42 dogs that came to autopsy is described.

2. A method is presented for its disruption.

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Studies on Antigens of Human Red Cells. II. Serological Properties of Rh Factor in Elinin.* (17771)

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In a previous report(1) a lipoprotein fraction of red cells containing the Rh and A and B factors was described and given the name elinin. A new term was required since it was shown that the post-hemolytic residue of red cells (stroma) could be separated into two fractions by solubility differences and that elinin was biologically distinct from S protein (stromatin) in that the latter did not contain Rh or A and B activity. Since it has not

been possible to obtain a further concentration of the Rh factor in elinin by chemical methods, purification of elinin by physical methods was necessary in order to characterize its chemical and biological properties and to prepare a uniform substrate for further attempts at fractionation. The details of the steps taken in the purification of elinin and the analysis of its chemical and physical properties are reported elsewhere(2,3). When uniform solutions of elinin[†] became available for animal and human injection we hoped to obtain the answer to two questions: (1)

2. Moscovitz, M., Calvin, M., and Evans, R. S., in press.

3. Danlicker, W. S., Moskovitz, M., Zimm, B., Calvin, M., in press.

[†] We have retained the name elinin for the purified fraction of red cells containing the Rh and A and B factors.

* This work was aided by the Rockefeller Foundation for Medical Research and the United States Public Health Services. The Cutter Laboratories, Berkeley, Calif., assisted in providing material.

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1. Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, v61, 416.

TABLE I.

Comparable Results in Inhibition Studies Using Anti-Rh₀ Serum with a Saline Agglutinin and a Serum with a Univalent or Blocking Antibody. Both sera showed an agglutinating titer of 1-64 in saline and serum dilutions.

Anti-Rh ₀ saline agglutinating sera		Saline diluent					
Dilutions of Elinin		1-2	1-4	1-8	1-16	1-32	1-64
Elinin Rh ₀ cells		0	0	0	++	+++	++++
" " " + 56°C		+++	+++	+++	+++	+++	+++
" Rh negative cells		+++	+++	+++	+++	+++	+++
Anti-Rh ₀ univalent sera		Serum diluent					
Elinin Rh ₀ cells		0	0	0	+	++	+++
" " " + 56°C		+++	+++	++	++	+++	+++
" Rh negative cells		+++	+++	+++	+++	+++	+++

Would elinin containing Rh activity prove antigenic in human subjects and (2) would it be possible to demonstrate neutralization of Rh antibodies *in vivo* in human subjects. While there is some evidence that the Rh factor in elinin is not antigenic when compared to whole red cells, we have as yet no evidence to indicate that injections of elinin in the amounts used so far will neutralize antibodies *in vivo*.

Methods and materials. We have continued to use the serum inhibition technic as the most reliable method of assay for the Rh factor in fractions from Rh-positive cells.

1. Assay of Rh activity in elinin; Anti-Rh' and anti-Rh₀ saline agglutinating sera and anti-Rh₀ univalent sera have been used with satisfactory results in the serum inhibition test. For serum inhibition tests the anti-Rh sera was diluted so as to show an agglutinating titer of 1-64 on further dilution, with a 2+ micro-agglutination of Rh positive cells at this dilution. The basic technic of the serum inhibition test has been described previously(1). Serial dilutions of antigen, which in this case is a 1% solution of elinin from Rh positive cells, were incubated with a constant strength of anti-Rh serum. The highest dilution of the elinin showing complete neutralization of the antiserum is referred to as the titer. If serum with a saline agglutinating antibody was used, saline was used as the diluent for the elinin, whereas normal human serum was employed if a univalent anti-Rh₀ antibody was used. The two types of sera gave comparable results with the same preparations of elinin as shown in Table I. Fresh

Rh positive cells of known specificity and avidity were added to the serum elinin mixture to demonstrate the presence of unabsorbed antibodies. Elinin from homozygous Rh negative cells (cde/cde) was used as a control in some observations. The usual control, however, was elinin heated to 56° for 5 minutes which destroys the Rh activity.

2. Preparation of elinin for injection. Since elinin could not be produced by sterile technic, it was necessary to filter the final preparation before use. A 1% solution of elinin in distilled water was found to pass quantitatively through a fritted glass filter, (ultrafine, Corning Glass Works) without loss of Rh activity. The addition of electrolytes prior to filtration produced aggregation of molecules and impaired filtration. After filtration sterility was tested and the preparation was made isotonic by addition of electrolytes. Elinin proved to be pyrogenic when injected intravenously into animal and human subjects, so attempts were made to reduce contamination with pyrogenic materials and to remove pyrogens present. It has not been possible to develop a sterile technic for the preparation of elinin in the super-centrifuge and ultracentrifuge, but contamination has been reduced to a minimum. Only freshly distilled water was used in the process of hemolyzing the cells and suspending the elinin. An attempt was made to reduce the pyrogenicity of elinin by the use of "decalso," a cationic exchange agent, following the procedure of Smith and Pennel(4), but

4. Smith, W. E., and Pennell, R. B., *J. Bact.*, 1947, v54, 715.

this method was not effective. However, the following treatment appeared to reduce to some extent the pyrogenicity of elinin for rabbits. An acetate buffer with an ionic strength of 1 (100 ml M sodium acetate plus 50 ml M acetic acid) was added to a sterile 1% solution of elinin in the ratio of 2 ml of buffer per 100 ml elinin solution. The pH of the buffer when diluted with water in this ratio was 5 at 20°C. Sterile technic was used in this procedure and the pH of the elinin after the addition of the buffer was not taken. As the elinin was kept in an ice bath and centrifuged at 0°C, the pH was probably slightly greater than 5. The elinin precipitated after adding the buffer and was allowed to stand 15 minutes. It was centrifuged at 2000 G for 5 minutes and the colorless water-clear supernatant fluid was removed by suction with a capillary pipette. Phosphate buffer ($\mu = 0.15$; pH 7.7 at 25°C) was added to the sediment to make the volume about equal to that of the original elinin solution. The sediment was mixed with the buffer by alternately drawing it into and expelling it from a pipette. It was allowed to stand 15 minutes, mixed again, and centrifuged at 2000 G for 15 minutes. The supernatant fluid was removed as above. The precipitated elinin was then ready to be injected. Four ml of this material in a concentration of 1% were injected into two rabbits; one had a rise in temperature of 0.5°C, the other manifested no change in temperature. Two ml in a concentration of 1% were injected intravenously into a normal female who was not sensitized to the Rh factor. She manifested a 0.5°C rise in temperature and complained of dizzy sensations 2 hours later. Four ml of the same material were injected into a male subject who had been sensitized to the Rh₀ antigen years previously by repeated transfusions. He developed a shaking chill, weakness, and his temperature rose to 39°C. This appeared to one of us a typical reaction to pyrogenic substances, and there was no evidence, such as asthmatic breathing or urticaria, that specific hypersensitivity was involved. As a result of the above reactions, it was decided to abandon the attempt to give large amounts of elinin intravenously to hu-

man subjects and to use the material available for intramuscular injection in approximately 8% concentration, which has not produced systemic reactions and has caused only transient local soreness.

Injection of elinin into animals and human subjects. Initial attempts to produce Rh antibodies by the injection of crude fractions of Rh-positive cells into rabbits and guinea pigs were not successful. Some of the 10 rabbits which received I.V. injections developed high titers of nonspecific red cell agglutinins (1-10,000), but none of the sera showed any specificity for Rh positive cells, either before or after adsorption with Rh negative cells. The guinea pig experiments were inconclusive largely because of a high mortality due to an unknown epizootic. When purified preparations of elinin became available, animal experiments were performed again as described below: *Rabbits:* Five-tenths of a ml was injected into the ear veins of each of 5 rabbits over a period of 5 weeks. Three injections were given the first 3 days of the first, third, and fifth weeks. Ten days after the last injection samples of blood were removed by cardiac puncture from each animal. *Guinea pigs:* Group 1—Six guinea pigs were injected intraperitoneally with 1 ml of a 1% elinin solution in 0.15 M NaCl at 48-hour intervals for a total of 4 injections. Four days after the last injection the animals were bled by cardiac puncture. Five ml were removed from each animal, and 2 of them died after the operation. Forty days after the last injection the four remaining animals were given 1 ml subcutaneously and 48 hours later were given 1 ml intraperitoneally. Twelve days later they were bled by cardiac puncture.

Group 2—Six guinea pigs were injected as in Group 1 except that a 17-day interval intervened between the fourth and fifth injection.

Group 3—Six guinea pigs were injected as in Group 2, using 1 ml of packed type O Rh₀ red cells instead of elinin. The cells were washed 4 times with 3 volumes of saline before injection. The red cells of a single donor were used and were removed by venous puncture the same day they were injected. The presence of Rh antibodies in the sera of the

sensitized animals was determined after removing the nonspecific agglutinins with Rh negative cells. The red cells used to absorb the sera were obtained from three Rh negative, MN donors. The red cells of the three donors were mixed and washed 4 times with 2 volumes of 0.15 M NaCl. The blood removed from the animals was allowed to clot in the refrigerator and the sera removed after centrifugation. The sera were frozen and kept in dry ice until tested. After the sera were thawed they were heated at 56°C for 30 minutes and tested within 24 hours. The sera were absorbed with equal volumes of packed red cells for 30 minutes at 37°C. The absorbed sera were tested with the Rh negative red cells used for absorption, red cells from a different Rh negative type O MN donor, Rh₁ type O red cells and Rh₀ type O red cells. The latter were those from the donor whose red cells were injected into the guinea pigs. Two absorptions were necessary before the sera from the rabbits and guinea pigs injected with elinin were completely absorbed; 4 absorptions were necessary with the sera from Group 3 guinea pigs. The absorbed sera from the rabbits and the guinea pigs of Groups 1 and 2 failed to agglutinate the Rh positive red cells; the absorbed sera from three of the guinea pigs of Group 3 agglutinated the Rh positive red cells and the absorbed sera of the others failed to agglutinate them. Aliquots of the unabsorbed sera from the guinea pigs of Group 3 were diluted 15 times with 0.15 M NaCl and absorbed and tested as above. Two absorptions were necessary to remove the agglutinins for Rh negative red cells. One of the sera gave slight agglutination with Rh positive red cells after absorption and the remainder were negative. This latter absorption procedure was repeated using a 30% albumin solution as the diluent instead of NaCl and no agglutination was obtained with any of the sera and Rh positive cells.

Humans: Elinin was injected into human volunteers who had been previously sensitized to the Rh factor. Five of the 6 subjects were women who had been sensitized during pregnancy and had been delivered of one or more infants with hemolytic disease of the new-

born. Two of the subjects were pregnant at intervals during the time of the injections. The sixth subject was a male, age 30, who had been sensitized by multiple Group O transfusions received during treatment of a combat injury. All the subjects had only univalent, Rh₀ (anti-D) type of antibodies in the serum. In 3 of the subjects the titer of antibodies was sufficient (1-64 or more) to demonstrate inhibition in the test tube by the Rh activity of the elinin injected. In all instances an attempt was made to compare the effect of injections of elinin with subsequent injections of whole Rh positive cells. Red cells were injected in the form of whole blood with 2.5% citrate. The blood was transfused without delay from donor to recipient. In one instance 1 cc of blood was given intravenously after producing complete hemolysis of the cells by mixing with 5 volumes of distilled water.

Calculations of yield of elinin from red cells indicate that 1 ml of 1% elinin is roughly equal in Rh antigen content to 3 ml of packed red cells. Serum inhibition studies with elinin and whole cells indicate that this calculation is correct. The injection of elinin with Rh activity into human subjects with anti-Rh antibodies was approached with considerable caution. Preliminary observations with intradermal and subcutaneous injections produced no immediate or delayed local reaction suggesting hypersensitivity. Thereafter intravenous and intramuscular injections were given without the occurrence of reactions suggestive of anaphylaxis, although intravenous injections were abandoned because of pyrogenic type of reactions. Following injection of elinin or whole blood, samples of serum for antibody studies were drawn at 7 to 10 day intervals. Antibody titrations were done with freshly drawn Rh₁ (CDe/CDe) cells. The serum was stored in the frozen state and antibody titrations were repeated when all sera pertaining to a single observation had been collected.

Results of human injection. In no instance was the injection of elinin into sensitized human subjects followed by a significant rise in antibody titer. These observations, which suggest a lack of antigenicity, are significant

TABLE II.

The Failure of Elinin with Rh Activity to Produce a Rise in Antibody Titer in Individuals Who Showed a Response to Subsequent Injections of Red Blood Cells.

Subject	Date	Antibody titer	Injection
W.M.	4/14/48	1-64	
	4/21	1-4	
	4/30	1-16	
	5/ 5	1-16	1.2 ml 1.5% Elinin I.V.
	5/14	1-32	1.0 ml Blood I.V.
	5/20	1-2560	
	6/16	1-8	
	9/30	1-2	3 ml 1.5% Elinin I.M.
	10/ 8	1-2	1.0 ml Blood I.M.
	10/15	1-3200	
	12/ 9	1-2	4.0 ml 2.5% Elinin I.V.
	12/20	1-2	
	4/15/49	1-512	1 ml hemolyzed blood I.V.
	4/22	1-512	
K.S.	1/13/49	1-64	1 ml 1.5% Elinin S.C.
	1/20	1-64	1 ml 1.5% Elinin I.V.
	1/28	1-128	
	10/13	1-32	1 ml Blood I.M.
	10/21	1-100	1 ml Blood I.V.
	10/27	1-1600	1 ml Blood I.V.
	11/ 3	1-16000	

TABLE III.

Antibody Titers Following Intramuscular Injection of 8% Solution of Elinin.

Subject	Date	Antibody titer	Injection 8-10% elinin
V.G.	Previous titers	1-4 to 1-128	
	2/21/49	1-32	5 ml I.M.
	2/23		5 ml I.M.
	2/24	1-32	
	3/ 1	1-32	
	4/19	1-64	
	4/22	1-64	10 cc I.M.
	4/26	1-64	
	4/30	1-64	
W.M.	6/ 4/49	1-1024	
	6/ 6	1-1024	10 cc I.M.
	6/10	1-512	
	6/15	1-512	
K.S.	3/28/49	1-400000	8 cc I.M.
	3/29	1-640000	9 cc I.M.
	3/30	1-640000	
	4/ 1	1-640000	
	4/11	1-640000	
	(Pt. delivered 9/49 of Rh negative baby)		
F.M.	6/ 4/49	1-1024	
	6/ 6	1-1024	10 cc I.M.
	6/ 7	1-2048	
	6/10	1-512	

in only 2 of the five individuals, since only 2 showed a rise in antibody titer following subsequent injection of red cells as shown in Table II.

Four of the 5 subjects were given intramuscular injections of elinin in amounts com-

prising the entire lot that could be produced in the ultracentrifuge in one week. In no subject was there a significant drop in antibody titer as shown in Table III.

Discussion. Our evidence to date indicates that elinin from Rh positive cells, while capa-

ble of neutralizing antibodies *in vitro*, is not antigenic when injected into animals or into human subjects, known to be capable of producing Rh antibodies. The failure to produce a rise in antibody titer in 2 human subjects who later responded to injections of whole red cells is a more convincing demonstration of lack of antigenicity than the failure to produce Rh antibodies in animals. It is interesting in this connection that one subject (W.M.) who showed a response to the injection of whole Rh positive cells failed to show a rise in antibody titer after the injection of freshly hemolyzed cells. Further observations are necessary to determine if the apparent loss of Rh antigenicity following hemolysis is partial or complete. The failure of the human subjects to show a drop in antibody titer following injections of elinin from Rh positive cells is not surprising. Calculations from serum inhibition studies indicate that 3 to 5 g of elinin will be needed to neutralize the circulating antibodies in an individual with an antibody titer of 1 to 64 if the relationship is stoichiometric *in vivo*. As yet the greatest amount given by I.M. injection is 1 to 1.5 g. In addition to the lack of quantity given we have no information as to the rate of absorption of elinin from an intramuscular depot or its antibody neutralizing activity when absorbed after exposure to tissue enzymes. The possibility that 3 to 5 g of elinin given intravenously would neutralize the circulating antibodies has not as yet been explored largely because of the difficulty of obtaining this amount of material and free from pyrogens. As stated

above, we have not been able to obtain an active fraction of crude preparations of elinin by chemical or enzymatic means. This has also been true of the purified preparations of elinin. We have not been able to repeat the work of Carter(5), in extracting an active substance from whole red cells by alcohol and ether. The preparations obtained by her method of extraction yielded uniformly negative results with our method of assay. We believe that the basic constitution of the Rh antigen is as yet unknown. * Observations of the complete inactivation of the Rh factor by trypsin suggest that the Rh hapten is very closely associated with a protein if it is not a protein itself.

Summary. 1. Elinin, a lipoprotein fraction of the red cell exhibiting Rh activity has been isolated in relatively pure form largely by physical methods.

2. Injection of elinin from Rh positive cells into animals has so far failed to yield a measurable titer of anti-Rh antibodies.

3. Injection of elinin into human subjects who have been sensitized to the Rh factor by pregnancy or transfusion failed to increase the titer of Rh antibodies in the serum. Two of the subjects injected showed subsequent response to injection of whole Rh positive cells.

4. Injection of 1 to 1.5 g of active elinin intramuscularly failed to effect a change in titer of circulating antibodies.

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Carbonic Anhydrase in Congenital Heart Disease.* (17772)

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Children with cyanotic congenital heart disease frequently tolerate relatively low de-

grees of oxygen saturation of the blood without any noticeable effects on the intelligence or on the state of consciousness when at rest. In connection with a study of the adaptive mechanisms involved, an investigation has been made of the possible role of carbonic anhydrase in the blood of such children. Car-

* This work was performed under a cooperative agreement between the University of Minnesota and the National Heart Institute, Public Health Service, Bethesda, Md.

TABLE I.
Carbonic Anhydrase Values in Patients with Different Cardiac States.

Cardiac status	Age range (years)	No. of cases	Carbonic anhydrase					Mean Hgb (g)	Mean RBC	Mean hematocrit
			Units mm ³	Max.	Min.	Mean	Stand. dev.	S.E.M.		
Normal	13/4-30	20	2.36	1.40	1.85	.27	.06	13.0	4.78	41
Newborn	NB-2 wk	13	0.69	0.10	0.41	.19	.05	15.4	3.86	48
Cyanotic congenital heart disease	3 $\frac{1}{4}$ -24	18	4.50	2.11	3.12	.72	.17	18.3	7.84	63
Acyanotic	1 $\frac{1}{2}$ -20	6	2.09	1.20	1.71	.19	.08	12.2		

bonic anhydrase was described first(1) as an enzyme which accelerates the reaction of carbonic acid to carbon dioxide and water. The same authors also showed that the enzyme is present in the hemoglobin of the red cells(2). Since the release of carbon dioxide and the uptake of oxygen are intimately related to each other, it was thought that investigation of this enzyme might throw light on such mechanisms. Using a slightly different technic than that used by ourselves, normal values for infants and children have previously been established by Stevenson(3).

Methods. The glass boat method described by Meldrum and Roughton(4) was used by us. The exact technic employed was that of Lambie(5). This consisted of using 2 ml of 0.2 molar phosphate buffer solution which was shaken vigorously with 2 ml of a 0.2 molar sodium bicarbonate in hydroxide solution, in an airtight chamber connected to a water manometer. This mixture was used as the control. With an identical setup, 0.2 ml of laked blood was added to the buffer side of the boat, and the increment in the velocity of evolution of carbon dioxide over the control was used as an index of the carbonic anhydrase activity of the blood. The enzyme unitage was calculated as described by Lambie(5). Our experiments were run at 15°C, using the same machine throughout the entire study. In each instance the blood was obtained by venipuncture and was stored until used in an oxalate tube at 4°C.

Results. The results of the studies in patients with various cardiac states can be seen summarized in Table I. Twenty patients with normal cardiac status ranging in age from 1 $\frac{3}{4}$ to 30 years of age had an average carbonic anhydrase value of 1.85 units. The standard deviation for this group was 0.273

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TABLE II.
Non-cardiac Infants.

Patient's No.	Age (wks)	Carbonic anhydrase (units/mm ³)	Hgb (g)	RBC (millions)	Hematocrit	Diagnosis
1	1	1.2	16.5	5.50	56	Spine bifida
2	2	0.77	10.8	4.63	35	Feeding problem
3	8	1.16	7.4	2.98	28	Rectovaginal fistula
4	8	0.80	8.5	4.60	29	Pyloric stenosis
5	20	2.31	12.4	5.15	40	Convulsions
6	20	1.14	10.3	5.58	35	Hydrocephalus
7	28	1.72	10.2	5.76	35	Normal

and the standard error of the mean 0.061. Thirteen newborn infants' sera were tested, the cord blood being used in each instance except one that was two weeks of age. The average carbonic anhydrase level in these newborn infants was 0.413, the standard deviation 0.188 and the standard error of the mean 0.052. In 18 patients with cyanotic congenital heart disease, varying in age from $\frac{3}{4}$ to 24 years, the average carbonic anhydrase level was 3.12, the standard deviation 0.716, and the standard error of the mean 0.169. Six individuals with acyanotic congenital heart disease varying in age from $\frac{1}{2}$ to 20 years had an individual carbonic anhydrase level of 1.71, a standard deviation of 0.190 and a standard error of the mean 0.078.

In an effort to determine the age at which the carbonic anhydrase content of the red cell changes, seven infants varying in ages from nine days to seven months were tested. These values may be seen in Table II.

Discussion. It has been suggested by Stevenson(3) that the low levels of carbonic anhydrase in premature and newborn infants is possibly a characteristic of fetal hemoglobin which is adapted to the intrauterine condition of low oxygen tension. We had thought previous to the present study that children with cyanotic congenital heart disease had possibly retained some fetal mechanism, such as fetal hemoglobin, that permitted them to utilize oxygen in the manner of the fetus. It would

appear from the results of this study that carbonic anhydrase is not the factor, as the patients with cyanotic heart disease have an elevated carbonic anhydrase which seems to be proportional to the red cell mass or hematocrit. Hodgson(6) has already demonstrated this relationship in other disease conditions. In our studies, when the mean value of 3.12 for carbonic anhydrase in the cyanotic heart group is corrected for the increased hematocrit, a value of 1.83 is obtained which is almost identical with that which we obtained in the normal group, namely 1.85.

It is apparent from these studies, that heart disease of the acyanotic type places no special load on the hematopoietic mechanism, and, therefore, the carbonic anhydrase level is the same as in normal individuals. It is also apparent from our data, as from those of Stevenson(3), that the carbonic anhydrase of the normal newborn infant is low in spite of the normal or increased hematocrit. Normal adult values are not reached until the infant is between 5 and 7 months of age, as shown in Table II. Stevenson(3) has made the comment that there is not a close correlation between the carbonic anhydrase and the hematocrit, red blood cell count or hemoglobin. Our data would indicate that this is true during the first year of life, whereas thereafter the carbonic anhydrase is definitely related to the hematocrit.

Summary. In an attempt to determine the possible adaptive mechanism in children with cyanotic congenital heart disease, the carbonic anhydrase level of the blood was determined in 18 patients of different ages with proved congenital heart disease of the cyanotic type. The values found were compared with those obtained on six patients with congenital heart disease of the acyanotic type, 13 normal newborn infants and 27 patients with no cardiac disease who varied in age from one week to 20 years.

It was found that the carbonic anhydrase as determined routinely was elevated in all

of the patients with cyanotic congenital heart disease as compared with that of patients with acyanotic congenital heart disease or with no heart disease. When corrected for the increased hematocrit, the blood carbonic anhydrase of the cyanotic patients did not differ significantly from that of the acyanotic subjects of similar ages.

The findings by Stevenson of excessively low values for carbonic anhydrase in the blood cells of normal newborn infants were confirmed.

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A Small Adjustable Renal Artery Clamp for Production of Chronic Hypertension in the Rat. (17773)

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In connection with studies concerning the production of arteriosclerosis in the coronary arteries, the need arose for an adequate method to induce chronic hypertension in the rat. For this purpose, a small, adjustable silver clamp, modeled after the larger Goldblatt clamp(1), has been designed and applied to the renal arteries of the rat(2). The clamp first employed measured 2 mm in length, 1.5 mm in width, and 2 mm in height. That finally used and illustrated in Fig. 1 is considerably smaller and measures 1.7 mm in height, 1.65 mm in length, 1.5 mm in width, and weighs 24 mg. It is made of sheet silver, 0.25 mm thick (0.01"), and nickel wire (0.85 mm diameter). A strip is rough cut to size for the casing (A). The edges are rounded and shaped with a fine stone to exact size and formed with a carefully machined punch die. On the top, a hole is centered, drilled, and tapped for the screw (B). Slots are cut with a saw 0.25 mm from the ends for the remov-

able plate (C). The screw is made of nickel wire which is threaded (6 threads/mm), and one end cut with a lathe and drilled to ac-

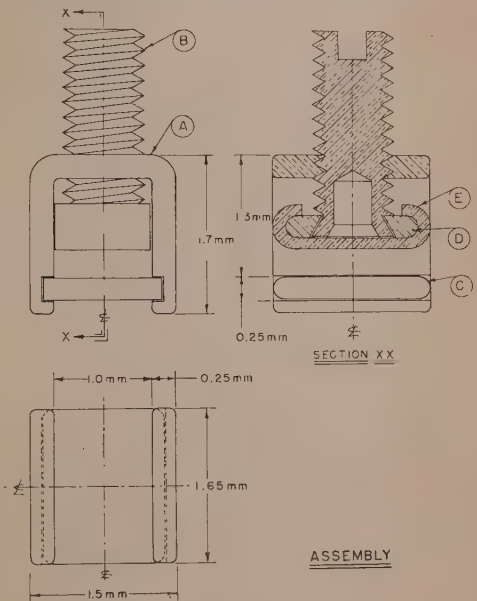


FIG. 1.

Assembly drawing of small, adjustable silver clamp.

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commodate the moving plate (D). A 2-mm length is slotted to take a small screw driver. It is then screwed into the casing. The movable compressing plate is drilled and the hole countersunk. It is then cut and shaped to size. All edges are rounded. The plate is then peened to the screw. A facing plate (E) of 0.12 mm silver is formed and bent to fit the moving plate. Finally, the removable bottom plate is cut, shaped, and finished.

The smaller clamps have been applied bilaterally at one operation with aseptic technique to the renal arteries of 11 rats. The screw of the clamp was turned to give complete occlusion of the artery and then turned back 1 to 1½ turns. This setting of the clamp was not subsequently altered.

Blood pressure, before and after operation,

was determined in the hind leg of the unanesthetized rat by a modification(2) of the method of Kersten *et al.*(3). Control blood pressures, before operation, varied from 100 to 125 mm Hg. Eight of the rats attained a blood pressure level of at least 160 mm Hg within a time period varying from 8 to 21 days postoperative. In 2 of the rats, such a pressure level developed somewhat later. One rat died postoperatively with massive infarcts of both kidneys. The pressures have remained elevated (160 up to a maximum of 220 mm Hg) for months in 9 of 11 rats.

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Occurrence of Renal Lesions in Guinea Pigs Given Parenteral PGA.* (17774)

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Human infants and guinea pigs on diets deficient in ascorbic acid and high in aromatic amino acids excrete p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids and small quantities of tyrosine in the urine(1,3). This abnormal excretion of metabolites is abolished by the administration of ascorbic acid(1,2,4). Since pteroylglutamic acid (PGA) increases

the oxidation of tyrosine by suspensions of liver from sulfa-treated rats(5) and because the excretion of phenolic compounds by patients with pernicious anemia is reduced by liver therapy(6) the effect of PGA on the urinary excretion of hydroxyphenyl compounds by scorbutic guinea pigs was studied(7). Daily doses of 5 mg of PGA were found to abolish the hydroxyphenyluria in these scorbutic pigs(7,8). An anatomical study was made of the animals used in these latter investigations and in other experiments devised to elucidate the problem of hydroxy-

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‡ Public Health Service Research Fellow of the National Institutes of Health.

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phenyluria in scorbutic pigs and its reversal by therapy with ascorbic acid, pteroylglutamic acid and other compounds. It is the purpose of this paper to describe renal lesions which were observed in some of these experimental animals.

Materials and methods. Albino guinea pigs weighing approximately 300 g were used. The basal diet consisted of heated (100° for 2 hours) skim milk powder, 30; fortified rolled oats, 39; wheat bran, 20; cottonseed oil, 8; sodium chloride, 1; and cod liver oil, 2. This diet contains approximately 0.8 μ g of PGA per gram. In the initial experiments tyrosine was added to the diets to a proportion of 5% and supplements of ascorbic acid, PGA, etc., were added in the quantities and for the time intervals indicated in individual experiments. The animals were maintained in individual metabolism cages and the urines collected for determination of total hydroxyphenyl compounds(8).

Results. The first animals studied consisted of groups treated as follows: basal diet plus 5% tyrosine; basal diet and tyrosine plus 25 mg of ascorbic acid daily; and basal diet plus tyrosine plus 5 mg of PGA daily. The ascorbic acid was given orally, the PGA parenterally. There was abundant hydroxyphenyluria in the animals on the unsupplemented basal diet with tyrosine but no appreciable hydroxyphenyluria in the animals which received the diet with tyrosine supplemented with either ascorbic acid or PGA. These animals were killed and studied without reference to their treatment. Although changes were observed in other organs, the chief findings were in the kidneys. On gross examination the kidneys were slightly enlarged and their surfaces were smooth, mottled and pale. Microscopic sections revealed that clusters of nephrons showed dilation of their tubules. These groups of nephrons were related anatomically to collecting tubules which were also dilated. Casts were seen in all varieties of tubules but they were most numerous in the larger collecting ducts and in the ducts of Bellini which were greatly dilated. Most of the casts were yellow and granular but some were homogeneous and hyaline. No necrosis of tubular epithelium was seen but

the lining cells of all the dilated tubules were flattened and basophilic. There were no glomerular changes.

Since the histologic studies were made without reference to the treatment that the animals had received, it was initially surmised that those kidneys showing the above-described lesion were from animals which had exhibited hydroxyphenyluria. On the contrary, however, the injured kidneys were from pigs which had received PGA and which had not shown hydroxyphenyluria. It was also of interest to note that no alterations were observed in the group treated with ascorbic acid. Because these lesions occurred only in the animals receiving PGA, it seemed advisable to determine the role played by PGA, tyrosine, and by the ascorbic acid deficiency. Consequently, groups of animals were placed on the basal diet plus 25 mg of ascorbic acid with and without added tyrosine. Half the animals in each group were given 5 mg PGA daily. It was impossible to determine presence of the biochemical lesion by observing hydroxyphenyluria which does not occur under these conditions. Autopsy studies showed that the renal lesion was present only in animals which had received PGA and that the presence or absence of tyrosine in the diet had no demonstrable effect on its development. Furthermore, the lesion in this series was identical with that observed in the earlier investigation in which all of the animals were scorbutic. The renal damage therefore, was associated with PGA administration and not due to either added tyrosine or to ascorbic acid deficiency.

In another study, 8 animals on the basal diet were given orally a suspension of 350 mg of tyrosine twice daily. Two of these animals were supplemented with 25 mg of ascorbic acid daily; 3 with 5 mg of PGA daily; and 3 were given no vitamin supplements. Tyrosine so administered was poorly tolerated. The food intake decreased to about one-third of usual except for the animals receiving ascorbic acid. All of these pigs lost weight rapidly and became moribund at the end of 2 weeks. When tyrosine was mixed in the diet in comparable amounts the majority of the pigs survived at least 3 weeks. No renal lesions were observed in the ascorbic

TABLE I.

Group No.	Daily supplement		Diet	Duration of exp. (days)	Renal lesions	Wt. changes (avg, g)
	PGA (mg)	Ascorbic acid (mg)				
I	0	0	Stock*	21	0	+112
	0	0	and	21	0	
	0	0	Cabbage	21	0	
II	5	0	Stock	21	0	+ 86
	5	0	and	21	0	
	5	0	Cabbage	21	0	
III	0	25	Basal	21	0	+ 29
	0	25	"	21	0	
	0	25	"	21	0	
IV	1	25	"	21	+	+ 42
	1	25	"	21	0	
	1	25	"	21	0	
V	3	25	"	21	+	± 9
	3	25	"	9	0	
	3	25	"	21	0	
VI	5	25	"	21	+	— 11
	5	25	"	21	+	
	5	25	"	21	+	
VII	1	0	"	21	+	— 57
	1	0	"	16	0	
	1	0	"	21	0	
VIII	3	0	"	21	+	— 82
	3	0	"	17	+	
	3	0	"	21	+	
IX	5	0	"	21	+	— 54
	5	0	"	21	+	
	5	0	"	3	+	
	5	0	"	11	+	

* Purina rabbit chow.

acid supplemented pigs and renal lesions were seen in all of the PGA treated animals. In the 3 animals receiving the tyrosine without supplementation one showed definite renal lesions while another showed questionable lesions. These animals are the only ones in which renal lesions have been observed in the absence of PGA administration. Inanition of this degree was not associated with PGA administration in the previous experiments. It seems improbable that inanition alone accounts for the appearance of the kidneys described above.

In all of the previous experiments, PGA had been administered parenterally in doses of 5 mg daily. The next series of experiments was designed to determine the amounts of PGA necessary for the production of the lesion and the effects of varying dietary conditions.

While the available evidence indicated that neither excessive tyrosine intake nor the scorbutic state was essential for the development of renal lesions, a further study of the role of the scorbutic state was made. To study the effect of ascorbic acid on the development of the lesion, the animals were run in 2 major series, one without ascorbic acid (Groups VII-IX) one with daily supplements of 25 mg of ascorbic acid (Groups III-VI). In each of these series were groups of 3 animals receiving by injection 1, 3, and 5 mg of PGA daily. The animals were maintained on this regime for 3 weeks. The results are indicated in Table I. Since other dietary factors might be involved in the phenomenon, studies were made simultaneously of 2 groups of 3 animals each (Groups I and II) which were fed a stock diet consisting of Purina rabbit chow

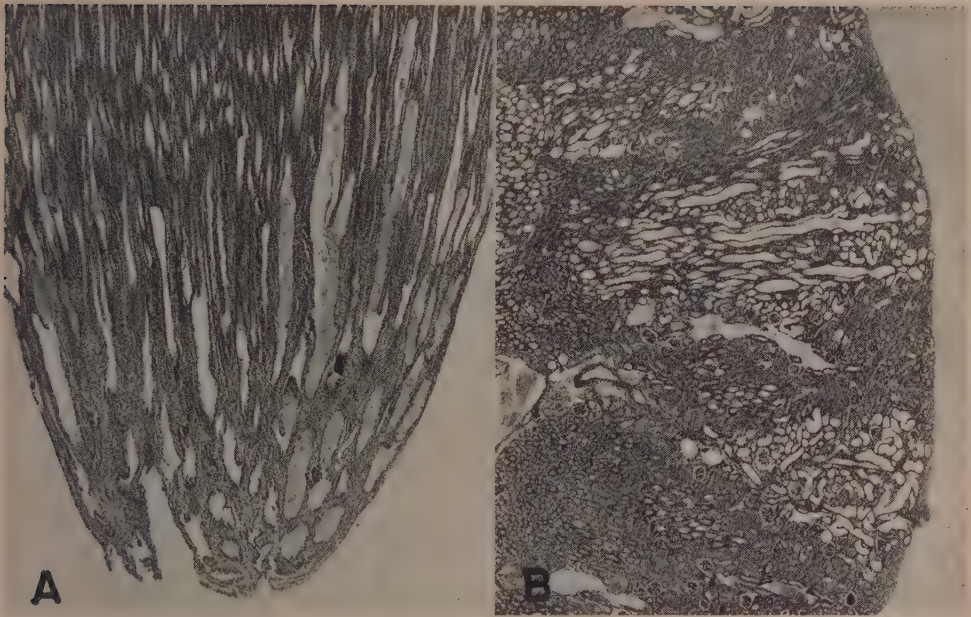


FIG. 1.

(a) Pyramid showing dilated, cast-filled ducts of Bellini. Hematoxylin and eosin $\times 30$.

(b) Cortex showing dilatation of tubules of nephron and collecting tubules. Hematoxylin and eosin $\times 30$.

plus cabbage *ad lib*. Three of these animals were given daily injections of PGA in amounts of 5 mg. In none of these 6 pigs did renal lesions develop in a 3 week observation period (Table I).

Three features of Table I deserve comment. First, both scorbutic and non-scorbutic guinea pigs developed kidney injury when injected with PGA. If any difference exists, the data indicate that the scorbutic animals are slightly more susceptible to the renal injury. The second point is that no lesions developed in the PGA-treated animals which were fed the stock diet and cabbage. The third point is the relationship between weight gain and renal injury. It will be noted that the lesions are least severe in animals which gained the most weight (those on the stock diet) and most severe in animals which lost the most weight (those on the experimental diet without ascorbic acid). It is also evident that administration of these quantities of PGA is associated with loss of weight or a reduction in the amount of weight gained.

In order to study the time required for the development of the renal lesions and to determine whether or not the lesions were reversible, another series of animals was given the basal diet plus tyrosine and 5 mg of PGA but without ascorbic acid or other supplements. Eighteen animals were used. Two animals were killed on the second, 4th, 6th, 8th, and 10th day after the start of the experiment. On the 12th day, the PGA was discontinued and animals were killed after 2, 4, 7, and 10 additional days. None of these animals exhibited renal lesions. Previous experience had shown that these lesions appeared after as little as 5 days treatment and that by two weeks all animals showed severe changes. In examining the details of this experiment in order to see where it might have differed from previous ones, it was determined that these animals were conditioned in the laboratory for several weeks prior to the start of the experiment whereas previous studies were made on pigs recently purchased from dealers. While they were held in our laboratory, they had

received a diet which was supplemented with unlimited amounts of cabbage. It will be recalled that in a previous experiment cabbage was used as a source of vitamin C in the diet which prevented the development of the renal lesions.

Discussion. The lesions described in this paper are similar to those seen by Harned, Cunningham, Smith, and Clark(9) in their experiments to determine the toxicity of PGA. These workers assumed that the casts are composed of PGA. The same assumption has been made by others(10,11). It is not unlikely that this is true in so far as the yellow casts are concerned, but the hyaline, eosinophilic casts are probably of different composition. We have no information on this point. The development of dilated atrophic tubules

is almost certainly due to obstruction by these casts.

The factors which govern the formation of these casts are not known; the influence of fluid intake on their formation and the manner in which a stock diet and cabbage prevent them remain to be studied.

Summary. 1. The administration of PGA to the guinea pig may lead to the development of casts in the renal tubules and to dilation of the obstructed nephrons.

2. The lesions may develop with parenteral doses of 1 mg of PGA daily for 21 days.

3. With daily parenteral doses of 5 mg of PGA the lesions may develop in 5 days.

4. The formation of these casts is not related to the feeding of tyrosine.

5. Scurvy is not a prerequisite for the appearance of the injury.

6. The lesions were not observed to occur on a stock diet containing cabbage. It appears that such a diet offers some protection against the development of the injury.

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Further Studies on Mechanism of Auricular Fibrillation.* (17775)

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The topical application of aconitine on the dog's auricle induces auricular flutter or auricular fibrillation. These arrhythmias are immediately stopped when the area of application is isolated from the rest of the auricles by a clamp, or cooled with the aid of a thermode. Removal of the clamp or cessation of the cooling leads to reappearance of the arrhythmias. The analysis of these tracings does not support a circus movement as the responsible mechanism(1-4). The presence of a focus of stimulus formation must be assumed. In ventricular flutter precipitated by the topical application of aconitine, cooling

also stopped the flutter and made sinus rhythm reappear for the duration of the cooling. Ventricular fibrillation, however, never responded to cooling. Evidence was advanced to show that with higher rates of ventricular flutter many centers became active in the ventricles. Therefore, cooling of the original center on which aconitine had been applied did not change the form of the arrhythmia(5).

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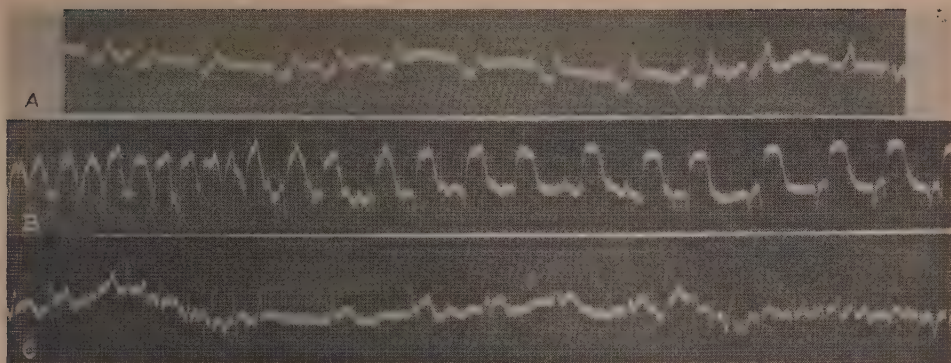


FIG. 1.

Fig. 1a shows temporary disappearance of auricular fibrillation during simultaneous cooling of sinus and A-V node. Fig. 1b shows the disappearance of auricular fibrillation caused by cooling simultaneously the sinus node and A-V node after opening of the right auricle. In Fig. 1c cooling of sinus node and A-V node abolishes fibrillation and A-V rhythm appears with inverted P waves seen after the QRS complexes.

We decided to study the effect of cooling on auricular fibrillation produced by methods other than aconitine.

Method. Dogs were used in all experiments. Nembutal was employed for anesthesia. The sternum was removed after artificial respiration had been started, and the pericardium was opened. In half of the experiments the vagus nerves were severed in the neck. When the vagus nerves remained intact prolonged fibrillation could be more easily produced. Auricular fibrillation was induced by 3 methods. 1. Faradization of the auricles was employed lasting for 30-60 seconds. This type of fibrillation rarely persisted long enough to permit experimental study. 2. In a few experiments prolonged fibrillation of the auricles appeared following rapid stimulation with rhythmic electrical shocks. They were applied with the aid of an electrodyne research stimulator with a frequency of 500 stimuli per minute. 3. The most effective method was the application of a small strip of filter paper moistened with a concentrated solution of acetyl choline (Roche), to the area of the sinus node. The solution used contained 0.1 g of the crystals dissolved in 2 cc of distilled water. With this method (6) prolonged auricular fibrillation always appeared. Satisfactory results were also

obtained when 0.05 cc of the solution of acetyl choline was injected sub-epicardially into the area near the head of the sinus node. Prolonged auricular fibrillation was necessary as only those experiments in which cessation of cooling allowed the fibrillation to reappear immediately could be evaluated. Cooling of the sinus node and of the auricular part of the A-V node was accomplished by the application of ice cold water in a glass tube, or by direct application of ice cubes on these areas. The latter method had the disadvantage that water from the melted ice spilled over the ventricular surface and deformed the ventricular complexes by depolarization currents. The A-V node was cooled in 3 ways. 1. By pressure with the thermode against the outer lateral wall of the right auricle, towards the lower wall of the right auricular septum in the area of the A-V node (coronary sinus node). 2. The heart was lifted from its bed by elevation of the apical area, and the A-V node was cooled through the thin walled coronary sinus vein at its orifice in the right auricle. It had been demonstrated before that warming this area invariably elicited coronary sinus rhythm with deeply inverted P waves (7). 3.

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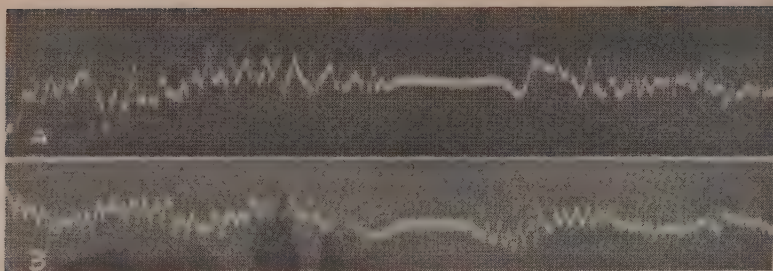


FIG. 2.

The tracings show temporary cessation of auricular fibrillation caused by cooling both the sinus node and the coronary sinus node through the coronary sinus vein. In the bottom tracing (Fig. 2b) flutter appears at the end of fibrillation and after the cooling thermode is withdrawn.

The superior and inferior vena cava were clamped and the auricle quickly opened so that the thermode could be applied on the area of the auricular part of the A-V node near the orifice of the coronary sinus vein. All electrocardiograms were taken in lead II.

Results. The experiments revealed that it was not possible to stop auricular fibrillation induced by any of the methods mentioned above, neither by cooling the stimulated area nor the area of application of acetyl choline. Even prolonged cooling of the whole sinus node was ineffective. The auricular fibrillation elicited by all three methods cited above always disappeared, however, for the duration of the cooling, when the area of the sinus node and of the auricular part of the A-V node were cooled simultaneously.

Fig. 1a, shows auricular fibrillation which had appeared after the application of acetyl choline to the area of the head of the sinus node. Cooling of the sinus node alone did not affect the fibrillation. Simultaneous cooling of the sinus node and of the area of the upper portion of the A-V node, through the wall of the right auricle, abolished the auricular fibrillation, and an A-V rhythm appeared. No P waves are visible. As soon as the fibrillation stopped, the cooling was discontinued and within a few seconds fibrillation started again.

In another experiment (Fig. 1b) auricular fibrillation was induced in the same way as in the experiment discussed above. The superior and inferior vena cava were clamped and the auricle was quickly opened. The

sinus node area and the area around the orifice of the sinus vein were simultaneously cooled; the fibrillation stopped and an A-V rhythm appeared with deeply inverted P waves preceding the QRS complexes. The ventricular complexes are deformed because the cooling of the sinus node was accomplished by means of a small ice cube and parts of the ventricular surface became depolarized by water from the melting ice.

Fig. 1c shows auricular fibrillation which appeared after the injection of about 0.05 cc of the solution of acetyl choline subepicardially into the area of the head of the sinus node. Simultaneous cooling of the sinus and A-V nodes abolished the fibrillation and an A-V rhythm with an inverted P wave between QRS and T replaced it. After interruption of the cooling, the fibrillation started again within 2-3 seconds. In this experiment, the A-V node area was cooled by pressing the thermode towards the wall of the right auricle in the area of the orifice of the sinus vein.

The tracings in Fig. 2 were obtained in 2 experiments in which auricular fibrillation was established by the application of acetyl choline on the sinus node. The heart was lifted from its pericardial bed by elevating the apex and the sinus node was cooled with one thermode while another thermode was applied to the area of the coronary sinus node (upper A-V node), through the terminal part of the sinus vein. In both experiments the cooling was stopped as soon as fibrillation disappeared. The tracings show the immediate reappearance of fibrillation with discontinuation of the cooling. In the lower tracing the heart was

lifted out of its pericardial cradle to such an extent that ventricular complexes are almost invisible. This tracing shows the slowing of the fibrillation to flutter before its disappearance, and the appearance of flutter changing quickly to fibrillation after the interruption of the cooling.

These results are the more remarkable because stretch and pressure tend to prolong the fibrillation or to establish it again once it disappears. Stretch and pressure were unavoidable during the cooling. The combined cooling was successful in 16 experiments on dogs weighing about 6.5 kg in which acetyl choline was used to initiate auricular fibrillation. Only in 3 large dogs, all weighing over 18 kg, were we unsuccessful in stopping the fibrillation induced by acetyl choline, by the combined cooling of the sinus and A-V nodes. In 2 experiments long lasting auricular fibrillation following faradization was abolished by the combined cooling and returned a few seconds after discontinuation of the cooling. The same observation was made in 2 experiments in which persistent auricular fibrillation appeared after the intravenous administration of pilocarpine and faradization. Whenever the fibrillation reappeared after it had been abolished by cooling, the renewed simultaneous cooling of the sinus node and the auricular parts of the A-V node re-established sinus rhythm. In one experiment prolonged auricular fibrillation had appeared after the subepicardial injection of Veratrine in the area near the head of the sinus node and in 3 experiments it followed rapid rhythmic stimulation of the auricle with 500 stimuli per minute. Combined cooling of the sinus and the A-V nodes abolished temporarily the fibrillation in each of these experiments.

Discussion. Auricular flutter and fibrillation elicited by the focal application of aconitine on the dog's auricle disappears when the focus is cooled. However, the auricular fibrillation caused by faradization, or by rapid rhythmic stimulation of the auricle, or by the focal application of acetyl choline are not stopped by cooling either the area of stimulation or the area of application of acetyl choline. This different response suggests that we are not justified in considering fibrillation

to be due to a single mechanism. Different forms with different mechanisms may exist as has been known for some time regarding extrasystoles. However, combined cooling of the sinus node and the auricular portion of the A-V node repeatedly abolished the fibrillation in 24 of 27 experiments. Only in 3 dogs with large hearts were we unsuccessful in changing the fibrillation to sinus rhythm by this method. Interruption of the cooling brought forth fibrillation again within a few seconds. Since in almost all dogs investigated by Lewis the circus wave moved up or down the sinus node, it is interesting to note that cooling of the whole length of the sinus node did not abolish the types of auricular fibrillation discussed in this paper. The re-appearance of the fibrillation once the cooling was interrupted is not compatible with a circus movement mechanism. A circus movement once arrested cannot later continue its way. A rapid stimulus formation in a center or centers is the other alternative. We consider it probable on the basis of these experiments that a rapid stimulus formation in one center leads to the same process in other centers, and thus, a rapid stimulus formation in the sinus node may also cause rapid stimulus formation in the A-V node. The possibility of rapid stimulus formation in the A-V node being responsible for auricular fibrillation has been stressed before. Haberlandt found the sinus beat regularly during auricular fibrillation caused by faradization of the frog's heart(8). Since the magnitude of the negative after potential is increased by rapid stimulus formation under certain conditions(9), the awakening of several centers is possible. The possibility that fibrillation is due to multifocal stimulus formation was often discussed before the circus movement theories were developed. It has been demonstrated that rapid stimulation in one ventricle may lead to the awakening of heterotopic centers in the other ventricle(5). The reason why the activation of other centers is so rare when aconitine is employed and is so common when fibrillation is caused

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by the measures discussed in this paper is not known. Aconitine, according to our results, causes auricular fibrillation by rapid stimulus formation in *one* center while in the other types of auricular fibrillation investigated by us more than one center is active.

Summary. Auricular fibrillation caused by electrical stimulation of the auricles or by the application of acetyl choline to the area of the sinus node is not stopped by cooling the area of stimulation or cooling of the site of application of the drug. In 24 of 27 experi-

ments, however, the simultaneous cooling of sinus and A-V nodes terminated the auricular fibrillation. Interruption of the cooling caused the auricular fibrillation to recur.

The experiments are evidence which tends to support the theory that in some forms of auricular fibrillation more than one center of rapid stimulus formation is active. They speak against the presence of a circus movement.

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Gonadectomy and Ovarian (Spleen) Grafts and Growth of Normal Male and Female Mice.* (17776)

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The more obvious end results of castration have long since been recognized as the complete failure of humans or the lower animals to undergo the body changes of normal puberty. Although individuals in the condition of acute gonad deficiency will continue to grow to or beyond adult proportions, the resulting state represents the persistence of a neutral or asexual form. It is in effect an expression of the growth impulse of the body in the absence of functioning gonads and their physiology. The effects of castration on body vigor lend themselves to objective experimental investigation. There is some degree of controversy among investigators concerning the effect which gonadectomy has on the growth of laboratory rodents. Slonaker(1) observed greater body weight in castrated

male rats although Stotsenburg(2), and Hatai (3,4) working with albino rats, reported that castration did not make any material difference in the body. Masui and Tamura (5) reported that castration did not affect growth of mice. Tang(6), Evans and Simpson(7), van Wagenen(8), Korenchevsky and Dennison(9), and Lawless(10), all of whom worked with rats, found that castrated male rats were lighter than their controls. Mice bearing active androgenic ovarian grafts do not assume a growth pattern comparable to that of normal males(Hill)(11).

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TABLE I.

Groups	"t"	Groups	"t"
1 + 2	9.93	2 + 4	2.42
1 + 3	4.98	2 + 5	5.01
1 + 4	6.64	3 + 4	1.00
1 + 5	5.06	3 + 5	1.04
2 + 3	3.01	4 + 5	2.32

Groups: 1. Control females. 2. Control males. 3. Castrate females. 4. Castrate males. 5. Castrate females—ovary grafts. "t"—Value of statistical significance.

Experimental. The present study was undertaken to consider the effect of castration on the body weights of laboratory mice and to determine to what extent the condition of ovarian and testicular deficiency would influence sex differences in growth. It likewise seemed of interest to compare any weight differences with the weights obtained from female mice bearing a graft of ovary in the spleen. The animals were all of the Swiss Albino strain and averaged between 75 and 85 days of age at the onset of the study. The mice were subdivided into 5 groups as follows, each group consisting of 20 mice: Group I, control females; Group II, control males; Group III, castrate females; Group IV, castrate males; and Group V, castrate females bearing an ovary graft in the spleen. Castration of Groups III and IV was completed when the animals were between 75 and 82 days of age. The ovary graftings of Group V were performed when the animals were from 85 to 95 days old. All animals were kept at normal room temperature and fed *ad libitum* with a standard diet.

The weights, recorded twice weekly, and reported in this experiment extend through a period of 40 weeks and are plotted graphically as indicated in Fig. 1. Computations of the "T" value of statistical significance (Snedecor) (12) were determined between the final average weekly weights of each of the 5 groups. The results obtained are summarized in Table I. The average body weight increase is summarized as follows: Group I, control females, showed an increase of 7.6 g; Group II, control males, an increase of 11.3 g; Group III, castrate females, 13.0 g; Group

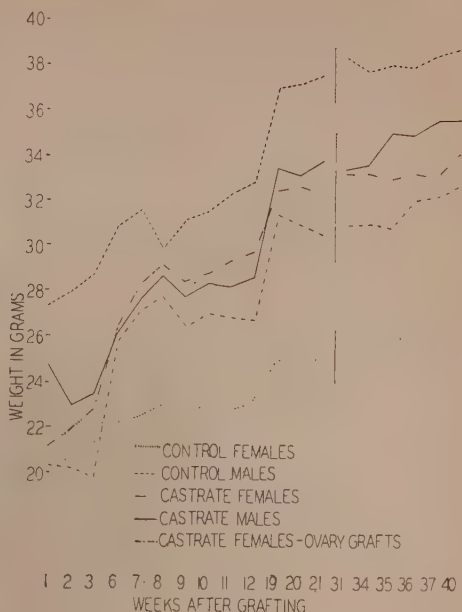


FIG. 1.

IV, castrate males, 11.0 g; and Group V, castrate females bearing ovary grafts, 12.5 g. Thus it is shown that castration of male mice has curtailed their gain in weight, while in females, the spayed animals as well as the animals bearing spleen grafts of ovaries are heavier than their normal controls. The "T" value is significant at 1% (Table I).

Tang(6), in a study of the sex difference in growth in gonadectomized albino rats found that castration of male rats did inhibit gain in body weight irrespective of the age of the animal at the time of castration. As judged by increase in weight, early castration and spaying had been no more effective in reducing the sex difference in growth than the later gonadectomy. Those animals which were gonadectomized at birth showed no apparent difference from the rest of the group. Growth in both castrated and normal males exceeded the growth rate of spayed and control females and the female mice bearing ovarian grafts. The removal of the ovaries in each of the 2 groups of experimental females affected an over-all percentage weight increase of approximately 61%, in comparison to an approximate 44% increase as a result of castra-

12. Snedecor, George W., Statistical Methods, The Iowa State Press, Ames, Iowa, 4th ed., 1946.

tion of the males. Sex difference (Fig. 1) cannot be entirely eliminated although it has been reduced by gonadectomy. Both groups of males still exceed in growth rate each of the 3 groups of females. This difference in growth rate may be due presumably to an inherent genetic factor. The body weight increase in the females bearing ovarian grafts was highly significant at the 1% level, when compared to the growth of the control and spayed females.

It has been demonstrated that no significant difference is obtained between the weights of normal females and those from grafted females bearing active androgenic grafts of ovaries in their ears (Hill)(11). The animals in this experiment had one ovary autografted into the spleen and the second ovary removed and discarded. Thus, any hormone that may have been produced by the ovary in its altered location would be transported directly to the liver via the hepatic portal system. Biskind(13), has conclusively demonstrated that natural and modified estrogens and androgens are inactivated in the liver. The estrogenic and androgenic hormones produced by the transplanted gonad, therefore, would presumably be inactivated, and the sex hormone in the systemic blood would approximate that of a nonimplanted castrate. Thus, it would appear that the significant difference in weight obtained from the females bearing ovarian (spleen) grafts and their normal controls could be attributed to the inactivation of the ovarian hormone. The growth of the normal males exceeded in every instance the growth of those females carrying ovary grafts in their spleen. These results are in agreement with the findings of Hill(11). Statistical evaluations of the comparisons indicated that the difference in weight between the grafted female mice and the control males was at least $2\frac{1}{2}$ times as great as that for the grafted animals and the castrated males.

Castrated male mice were found to be lighter than their controls, the difference being sig-

nificant at the 1% level. Freudenberger and Clausen(14) have postulated that it is oestrin that has the inhibitive effect on body growth in the female rat. The difference between the male and female controls in this experiment was significant, while the difference between the male and female test animals—*i.e.*, the gonadectomized animals, was barely significant at the 1% point.

A comparison of the grafted animals with the female controls, the male controls, and the castrate males, indicates a difference highly significant at the 1% level. Just as an intermediate type of growth curve was established for gonadectomized male and female rats against their controls (Tang)(6), so too, it would appear that a similar type of curve also results between the control and spayed female mice as compared to those animals bearing autograft ovaries in their spleen.

Summary. Castrated male mice grew at a slower rate than their normal controls, while the spayed females grew faster than their normal controls. The removal of the gonads reduced the sex difference in body weight. Those animals bearing grafts of ovaries also grew at a faster rate than their normal controls, the difference being significant at the 1% point. Although their growth curve approximated that of the spayed animals, at no time did it exceed it. The growth curve assumed by the weights of the grafted female mice did not parallel that of either the normal males or the castrated males. The differences in the body weight of the grafted females compared with the normal males and the castrated males were significant at the 1% level, the males being heavier in both cases.

My sincere appreciation and gratitude to Doctor R. T. Hill (now at the University of Miami, Medical Research Unit, Veterans Administration Hospital, Coral Gables, Florida) for his constant interest and assistance during the entire course of this study.

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Effect of Orally Administered Potassium Iodate on Blood Sugar Response to Thiourea.* (17777)

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The profound influence which goitrogenic drugs may exert on carbohydrate metabolism has recently been demonstrated. DuBois, Holm and Doyle(1) observed a marked hyperglycemia with concurrent depletion of liver glycogen 2½ hours after intraperitoneal injection of alpha-naphthylthiourea (ANTU) in adult rats. Previous administration of large doses of cysteine (1000 mg per kg) afforded protection against lethal doses of the goitrogen. Griesbach, Kennedy and Purves(2) have demonstrated that iodide injection in doses of 1.30 mg every fourth day into rats protected them against thiourea toxicity. Byerrum(3) likewise demonstrated the effectiveness of iodine, either as Lugol's solution or as potassium iodide, in protecting rats against the toxic effects of ANTU.

The purpose of this experiment was to determine the effect of orally administered potassium iodate on the blood sugar response of rats injected intraperitoneally with thiourea.

Experimental. 24 rats of both sexes of the Wistar strain were separated into 2 groups

of 12 each. Rats of Group I (Table I) ranging in weight from 148 to 398 g were bled from the tail and glucose concentration determined by the Folin-Wu micro method, after which thiourea (10 mg/kg) was injected intraperitoneally. A second blood sugar determination was made 2½ hours later.

The 12 rats in Group II (Table II) ranging in weight from 147 to 392 g received 0.2% potassium iodate in their drinking water *ad libitum* for 2 days, after which blood sugar determinations were made as in Group I. Then thiourea was injected intraperitoneally as in Group I (10 mg/kg) and a second blood sugar determination made after 2½ hours as before.

Results. The 12 rats in Group I receiving only thiourea showed an average rise in blood sugar of 91.0 mg per 100 cc 2½ hours following injection. Rats in Group II which had been fed 0.2% KIO₃ when injected with thiourea at the same dosage level as those in Group I showed an average blood sugar increase of only 14.6 mg per 100 cc. Statistically the results are significant, the *t*-value

TABLE I.
Effect of Thiourea on Blood Sugar Level of 12 Non-fasted Rats. Second blood sugar reading (B) was taken 2½ hr after rats received 10 mg/kg thiourea intraperitoneally.

Sex	Wt g	Blood Sugar (A) mg/100 cc	Blood Sugar (B) mg/100 cc	Difference (B-A)
M	379	90	165	75
M	387	90	187	97
M	398	86	165	79
M	358	86	117	31
M	148	83	225	142
F	322	86	165	79
F	372	90	186	96
F	348	79	165	86
F	324	66	140	74
F	317	79	300	221
F	342	109	140	31
F	168	105	186	81
			Avg Blood Sugar Incr.	91

* This work was aided by a grant from the Purdue University Alumni Research Foundation.

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TABLE II.
Effect of Thiourea on Blood Sugar Level of 12 Non-fasted Rats Previously Fed KIO₃. Sugar reading (B) was taken 2½ hr after rats received 10 mg/kg thiourea intraperitoneally.

Sex	Wt g	Blood Sugar (A) mg/100 cc	Blood Sugar (B) mg/100 cc	Difference (B-A)
M	383	63	79	16
M	392	59	86	27
M	366	76	90	14
M	333	90	93	3
M	319	63	97	34
F	285	93	105	12
F	315	105	122	17
F	186	102	97	-5
F	147	69	86	17
M	193	83	97	14
M	201	90	97	7
M	150	73	93	20
			Avg Blood Sugar Incr.	14.7

of 5.16 indicating less than one chance in a thousand of the results being accidental.

Summary. Oral administration of potassium iodate for a 2-day period in drinking

water prevented the hyperglycemia which followed thiourea injection in rats.

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Microbial Synthesis and Multiple Nature of *Lactobacillus bulgaricus* Factor. Possible Role in Chick Nutrition.* (17778)

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Recently Williams, Hoff-Jorgensen and Snell(1) reported that many natural products contained a previously unrecognized factor necessary for the growth of several strains of *Lactobacillus bulgaricus*. An assay method was described in which yeast extract was used as a standard of comparison and given an arbitrary potency of one unit per mg. Application of this assay method to culture filtrates of several microorganisms revealed that the *L. bulgaricus* factor (LBF) was generally synthesized and excreted in part into the medium by organisms which did not require it preformed (Table I). Such

culture filtrates were in some cases appreciably more potent sources of LBF than is yeast extract. The richest source of LBF yet found is the culture filtrate from *Ashbya gossypii* NRRL-1056, which on the dry basis is 3 to 5 times as active as yeast extract. LBF is also produced by *A. gossypii*, although to a lesser extent, in a synthetic medium containing glucose, amino acids, salts and vitamins. Fractionation characteristics of LBF(1) suggested that more than a single active entity might occur naturally. To check this possibility, culture filtrates of *A. gossypii* were chromatographed on paper strips in a manner similar to that described by Winsten and Eigen(2). Water-saturated

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TABLE I.
Synthesis of LBF by Microorganisms.

Organisms	Type of medium	Units of LBF in growth medium per ml	per mg solids
<i>Candida crusoides</i> (arborea)	Synthetic*	6	0.6
<i>Mycotorula lipolytica</i>	"	7	0.7
<i>Saccharomyces cerevisiae</i> Y-30	"	6	0.6
<i>Torula utilis</i>	"	6	0.6
<i>Aspergillus niger</i>	"	12	0.2
<i>Penicillium chrysogenum</i> 1380	"	15	1.7
" "	Corn-steep, glucose†	42-72	1.5-2.7
<i>Ashbya gossypii</i> NRRL-1056	Grain stillage, animal stick liquor, glucose†	75-125	3-5
<i>Lactobacillus pentoaceticus</i>	Yeast water, glucose†	24	1.0

* The synthetic medium varied slightly from one organism to another, but contained ammonium sulfate as the nitrogen source and glucose as the energy source in all cases. Appropriate inorganic salts and vitamins (where required) completed the medium, which was free of LBF. All organisms were incubated 3-4 days, and the activity determinations carried out on the culture filtrates.

† These crude media contained only small amounts (4 to 9 units per ml) of LBF before fermentation. Stillage is an aqueous residue of alcoholic fermentation; animal stick liquor is an infusion of slaughter house wastes which was obtained from Armour and Co.

TABLE II.
Comparative Rf Values of *Lactobacillus bulgaricus* Growth Factors Found in Yeast Extract and *Ashbya gossypii* Fermentation Products.

	Rf values				
	0.04	0.2	0.38	0.55	0.9
Difco yeast extr.	+	+	+	+	+
<i>Ashbya gossypii</i> culture filtrate	+	+			+
LBF conc. from <i>A. gossypii</i> culture filtrate					+

butanol was the mobile phase. When the strips were developed on agar plates seeded with *L. bulgaricus*, at least 3 zones of growth were observed. Chromatographs of yeast extract revealed 5 zones of which 3 were identical in Rf values to those contained in *A. gossypii* liquor. With LBF concentrated from fermentation liquor by a modification of an earlier described procedure(1) which involved adsorption on charcoal and elution with hot butanol, the same chromatographic procedure gave only one significant zone of growth, this having an Rf value of approximately 0.9. Rf values for the active substances present in the 3 materials are shown in Table II. It thus becomes apparent that several forms of the growth factor occur naturally, and that only one of these is concentrated by the procedure utilized above.

To determine the possible role of LBF in chick nutrition a concentrate of the factor was prepared(1) containing 108 units of activity per mg. By *L. leichmannii* assay this preparation was free of vitamin B₁₂.

The chicks used were from the Hiram Walker flock of Hampshire males mated to Barred Rock females. The maternal diet, which resulted in chicks of low animal protein factor(s) storage, was an all vegetable protein base. In this experiment, the hatchability of fertile eggs was 46%.

Day-old chicks from the above hens were placed on the following basal ration: ground yellow corn 43.2, pulverized oats 10.0, wheat bran 3.0, wheat standard middlings 5.0, mineral mixture [steamed bonemeal 100, ground limestone 50, manganese sulfate 1.5] 3.0, iodised salt 0.5, soybean meal 35.0 and vitamin supplement [Viadex (5000A-1000D) 2,000, riboflavin 4, choline chloride 250, wheat standard middlings 1746] 0.3. To this ration the concentrate of LBF was added singly and in combination with fish solubles and crystalline vitamin B₁₂ as shown in Table III. All chicks were reared in batteries with raised wire floors and with free access to water. The average weight gains after a 6-week period (Table III) show that the growth

TABLE III.
Six Weeks Gains of Chicks Fed Crystalline Vitamin B₁₂, Sardine Fish Solubles and LBF Concentrate.

Supplement (per 100 g of feed)	No. chicks		Avg 6 week responses (males + females)		
			g gain in wt	Gain over controls	
	Started	Died		g	%
None	34	6	217	—	—
LBF conc. (.06 g)	34	3	242	25	11.5
Fish solubles (1 g)	34	4	440	223	103
LBF conc. (.06 g) and fish solubles (1 g)	34	1	483	266	123
Fish solubles (3 g)	34	6	519	302	139
LBF conc. (.06 g) and fish solubles (3 g)	34	2	546	329	152
Crystalline vit. B ₁₂ * (3 µg)	34	3	504	287	132
LBF conc. (.06 g) and crystalline vit. B ₁₂ (3 µg)	34	1	515	298	137

* Kindly furnished by E. R. Squibb and Sons.

rate of all groups receiving the LBF concentrate was increased. It is also of interest but of uncertain significance that death losses were lower where the LBF concentrate was included in the diet. Groups receiving the concentrate appeared better groomed than those that did not receive it, a factor which is not reflected in the more objective data recorded in Table III. Two other trials in which a concentrate of LBF was added to the above basal ration gave substantially the same growth response and general improvement in the appearance of the chicks.

The weight of recent evidence indicates that crystalline vitamin B₁₂ does not fully duplicate the growth effects of crude sources of the "animal protein factor" when added to rations based upon vegetable proteins(3). The results of this and similar experiments are in accord with this view. Since 3% of

sardine fish solubles consistently produces the maximum growth obtainable with this supplement in the basal ration used the further increase resulting from the addition of the concentrate of LBF points to the existence of a factor in this concentrate which promotes growth and seemingly is inadequate in fish products. Likewise, the somewhat lower response to vitamin B₁₂ and to vitamin B₁₂ combined with LBF concentrate in comparison to fish solubles indicates that the latter carry at least one growth-promoting substance other than vitamin B₁₂.

Conclusions. These studies substantiate and extend an earlier report regarding the wide distribution of the *Lactobacillus bulgaricus* factor in nature. In addition they reveal the possibility that this factor may be required for animal growth. Investigations on the biosynthesis, purification, and nutritional significance of this factor are being continued.

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Influence of Alpha-Naphthylthiourea (ANTU) on Iodine Metabolism.* (17779)

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In previous studies we demonstrated(1,2) that pre-treatment of rats with inorganic iodide administered either in the diet or parenterally afforded this species protection against several lethal doses of alpha-naphthylthiourea (ANTU). Other investigators subsequently obtained(3,4) similar results with rats. An extension of our initial studies revealed that large quantities of iodide provided guinea pigs(5) with no significant increase in resistance toward ANTU and the protective effect of iodide could not be demonstrated in thyroidectomized rats(2). The mechanism whereby iodide protects against the acute toxicity of ANTU has not been elucidated. DuBois and Erway(6) found that iodide diminished the strong inhibitory action of ANTU on tyrosinase and although this effect was not considered as an explanation for the prophylactic action of iodide against ANTU it suggests that iodide might protect some oxidative reaction from inhibition by ANTU. It had been shown that sublethal doses of thiourea derivatives other than ANTU(7,8)

prevent the uptake of radioactive iodine by thyroid tissue. Thus, there exists a considerable amount of evidence suggesting that acute as well as chronic ANTU poisoning may involve interference with iodine metabolism. We, therefore, undertook an investigation of the effects of ANTU on the iodine metabolism of rats and guinea pigs and the present communication contains the results of iodine measurements on the tissues of normal and ANTU-poisoned animals receiving diets containing normal and high quantities of iodine. The influence of ANTU on the rate of uptake of iodine by the tissues of rats and guinea pigs was also ascertained using radioactive iodine (I^{131}) in tracer quantities in order to observe whether there was a correlation between the effects of ANTU on iodine uptake and the species susceptibility to the rodenticide.

Materials and methods. Sprague-Dawley rats weighing from 200 to 300 g and guinea pigs weighing 300 to 500 g were used in this study. In experiments concerned with the effect of ANTU on the iodine content of the tissues rats were fed Purina Laboratory Chow and guinea pigs were maintained on Purina Rabbit Chow. The high-iodine diet employed for studies with rats consisted of the synthetic 18%-casein diet used in our previous studies (2) with the addition of 5.3 mg of iodide as potassium iodide per g of diet. When low-iodine diets were fed to rats the same ration was employed with potassium iodide omitted from the salt supplement. Since guinea pigs did not readily accept the synthetic diet or Purina Rabbit Chow containing increased quantities of iodide they were fed a normal diet and iodine was administered in the drinking water at a concentration of 2.85 mg/ml. Rolled oats constituted the low-iodine diet for guinea pigs. ANTU was dissolved in propylene glycol and

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administered intraperitoneally with the strength of the solution being adjusted so that no animal received an amount of propylene glycol greater than 0.5% of its body weight. For studies with radioactive iodine a carrier-free solution of I^{131} , obtained from the Oak Ridge National Laboratory, was employed. The dose usually given was 10 microcuries administered intraperitoneally. Animals were fed a low-iodine diet for several days before administration of the isotope because this procedure resulted in a higher and more constant uptake of I^{131} by the tissues. At intervals after the administration of I^{131} the animals were sacrificed and the tissues were homogenized(9) in distilled water and diluted so that 2 ml of homogenate contained radioactivity within the range of accurate measurements. The I^{131} was measured using a Geiger-Muller apparatus with an end-window mica counter. For measurements of the iodine content of rat and guinea pig tissues animals were sacrificed by decapitation and 30% homogenates of the tissues were prepared. Total iodine was determined by the method of Perkin *et al.*(10). Separation of organically-bound from inorganic iodine was performed by the procedure of Palmer, Leland, and Gutman(11) as modified by Salter(12).

Experimental. The effect of ANTU on the iodine content of rat and guinea pig tissues. The iodine content of tissues from rats receiving a normal diet has been reported previously(12,13) and our values are essentially in agreement with those of other workers. The iodine content of tissues from 11 normal and 5 ANTU-poisoned rats is shown in Table I in which the distribution of iodine between the organic and inorganic fractions is given as well as the values for total iodine. These

TABLE I.
Effect of ANTU on Total, Inorganic and Organic Iodine of Rat Tissues.

		Normal	6 hr after 10 mg/kg ANTU
Blood mg%	Total	.012 $\pm$.0038*	.0216 $\pm$.0022
	Inorg	.0046 $\pm$.0003	.0137 $\pm$.0057
	Org.	.0091 $\pm$.0019	.0130 $\pm$.0042
Lung mg%	Total	.0482 $\pm$.0085	.0643 $\pm$.0071
	Inorg.	.0102 $\pm$.0057	.0208 $\pm$.0080
	Org.	.0401 $\pm$.0040	.0258 $\pm$.0068
Liver mg%	Total	.0195 $\pm$.0012	.0191 $\pm$.0037
	Inorg	.0111 $\pm$.0037	.0066 $\pm$.0010
	Org.	.0133 $\pm$.0022	.0089 $\pm$.0015
Thyroid mg%	Total	129.2 $\pm$ 13.2	66.7 $\pm$ 6.3
	Inorg.	4.36 $\pm$ 1.1	1.6 $\pm$ 0.8
	Org.	104.1 $\pm$ 13.3	56.8 $\pm$ 13.6

* $\pm$ values equal 2 stand. dev. from the mean.

determinations demonstrated that the outstanding effect of acute ANTU poisoning on the iodine content of rat tissues was a decrease in thyroid iodine to about 50% of the normal value. Coincident with the decrease in thyroid iodine there occurred an increase in both the organic and inorganic iodine of the blood and an increase in the inorganic fraction in lung tissue accompanied by a decrease in organic iodine. The changes in the distribution of iodine in the lungs were interesting because pleural effusion and pulmonary edema are the most pronounced pathological manifestations of ANTU poisoning.

The iodine content of tissues from normal and ANTU-poisoned guinea pigs was also examined. The total iodine in the thyroid tissue of normal guinea pigs was 39.2 mg% which is approximately 30% of the normal value for rats. Seven hours after the administration of 200 mg/kg of ANTU the total iodine of guinea pig thyroids was 59.4 mg% which represented an increase on a weight basis. However, paralleling this increase in total iodine there was a decrease in thyroid weight to about one-half of the normal value. Thus, the thyroid iodine per animal showed an overall decrease after ANTU. A dose of 200 mg/kg of ANTU in guinea pigs is comparable to 10 mg/kg in rats in terms of the LD-50 of ANTU for the two species.

The effect of increased dietary iodine on the concentration of iodine in tissues. Previous studies in this laboratory(1,2) have shown

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TABLE II.
Influence of High-Iodine Diet on the Iodine Concentration of Rat Tissues.
(5.3 mg I/g diet fed for 5 days)

Iodine Fraction	Blood mg%	Lung mg%	Liver mg%	Thyroid mg%
Total	4.98 $\pm$.61*	4.49 $\pm$.63	1.33 $\pm$.66	270 $\pm$ 25
Inorg.	3.43 $\pm$.50	3.32 $\pm$.58	.87 $\pm$.35	36 $\pm$ 6
Organic	1.19 $\pm$.30	.52 $\pm$.29	.35 $\pm$.10	216 $\pm$ 12

* $\pm$ values represent 2 stand. dev. from the mean of 8 measurements.

that increasing the dietary iodine causes a marked increase in the ability of normal rats to withstand ANTU while thyroidectomized rats received no protection from high-iodine diets(2). These findings indicated that the thyroid gland was involved in the protective effect of iodide against ANTU. Iodine determinations were, therefore, made on blood, lung, and liver from normal and thyroidectomized rats fed the high-iodine diet for 5 days. Iodine measurements were also performed on the thyroids of the normal animals receiving increased dietary iodine. The results of iodine measurements on the tissues from normal rats fed a high-iodine diet for 5 days are shown in Table II in which each value is the average for at least 5 animals. It may be noted from a comparison of the data in Table II with that presented in Table I that a pronounced increase in tissue iodine resulted from the feeding of high-iodine diets for 5 days during which time the average daily intake of iodine was 210 mg/kg. The inability of thyroid tissue to increase its iodine content to as great a percentage as other tissues under similar conditions, as shown in Table II, has been explained by Wolff and Chaikoff(13,14) on the basis that increases in blood iodine inhibit the enzymes or other mechanisms concerned with the incorporation of iodine into organic form.

A comparison of the iodine concentrations in tissues from normal and thyroidectomized rats fed the high-iodine diet revealed no significant differences in the quantity of iodine in the lung, blood, or liver of the two groups. These findings indicated that the failure of iodine-fed, thyroidectomized rats to exhibit resistance toward the acute toxicity of ANTU is not due to a difference in the ability of the

tissues to retain iodine thus providing further indications that the thyroid gland is responsible for the prophylactic effect of iodine against acute ANTU poisoning in rats. A group of guinea pigs was given iodide at a concentration of 2.85 mg/ml in the drinking water. At the end of a 5-day feeding period, during which time they received an average daily dose of 168 mg/kg of iodide, these animals failed to exhibit any increased resistance toward ANTU. The LD-50 was approximately 200 mg/kg for both normal and iodine-fed groups. Other similarly treated guinea pigs were sacrificed and total thyroid iodine was determined. During the 5-day feeding period the average daily iodide consumption was 182 mg/kg, and, although the total thyroid iodine increased almost 60%, an attendant decrease in thyroid weight caused the total thyroid iodine per animal to remain approximately normal. This differed from the results obtained with rats in which total thyroid iodine doubled after feeding high-iodine diets for 5 days.

Effect of ANTU on the uptake of radioactive iodine by rat and guinea pig tissues. To obtain further information on the influence of ANTU on iodine metabolism measurements on the rate of uptake and the retention of I^{131} were carried out on normal and ANTU-poisoned rats. Two groups containing 5 rats each were given 10 microcuries of I^{131} . Five hours later both groups were sacrificed and it was observed that ANTU strongly inhibited the uptake of I^{131} by the thyroid gland of rats. An average of 9.8% of the injected dose of I^{131} was found in the thyroids of normal rats as compared with only 1% for the ANTU-poisoned group. There was no significant difference in the quantity of I^{131} taken up by the other tissues from normal and ANTU-poisoned rats. Thus, the lungs of both groups

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contained an average of 0.06 microcuries of I^{131} /g while liver contained 0.037 microcuries/g. We also measured the uptake of I^{131} by the tissues of ANTU-poisoned guinea pigs because of the resistance of this species toward ANTU. When guinea pigs which were previously fed an iodine-deficient diet for 1 day were given 10 microcuries of I^{131} an average of only 1.36% of the injected dose was found in the thyroids. The uptake of I^{131} by guinea pig thyroids was therefore only about 0.1 as great as for rats under comparable conditions. Five hours after the intraperitoneal administration of 10 mg/kg of ANTU followed immediately by 10 microcuries of I^{131} the thyroids of guinea pigs contained 0.45% of the injected dose. Administration of 150 mg/kg of ANTU to guinea pigs resulted in only 0.06% of the injected dose being taken up by the thyroids in 5 hours. This dose of 150 mg/kg of ANTU given to guinea pigs is comparable to 10 mg/kg in rats and it produced a similar inhibition of the uptake of I^{131} . Thus, there was a correlation between the toxicity of ANTU and its inhibitory effect on the uptake of iodine by the thyroids of the two species.

Discussion. The results of these studies demonstrated that ANTU reduces the thyroid iodine of rats and guinea pigs with much higher doses being necessary to decrease the thyroid iodine of the latter species which is much more resistant than rats toward ANTU. The normal thyroid iodine value for guinea pigs was 39.2 mg% as compared with 129 mg% for rats. In rats lethal doses of ANTU produced a decrease in thyroid iodine and did not alter the size of the gland while in guinea pigs the thyroid weight decreased to about one-half the normal value after lethal doses of ANTU. The differences in the normal thyroid iodine values for the two species and in the response of the thyroid to ANTU is interesting in view of the marked difference in toxicity of ANTU for the two species.

Our previous experiments had shown(1,2) that rats acquire resistance to several times the lethal dose of ANTU when fed high-iodine diets for short periods of time while guinea pigs do not develop added resistance when fed iodine(5). It appeared from the

results of our previous experiments(2) that the thyroid gland was involved in the protective effect of iodine because thyroidectomized rats were not protected by iodide. The iodine determinations presented in this communication seem to offer further evidence in support of the idea that the protective action of iodide is mediated through the thyroid gland of rats. The iodine analysis on tissues from normal and thyroidectomized rats demonstrated that thyroidectomy does not influence the quantity of iodine in the tissues of iodine-fed animals. Therefore, the only apparent difference between normal and thyroidectomized rats fed a high-iodine diet in so far as the iodine concentration of the tissues is concerned was the presence of a high quantity of thyroid iodine in the unthyroidectomized rats. It appears that this increase in thyroid iodine from 129 mg% to 260 mg% is in some manner responsible for the elevation of the LD-50 of ANTU from 6.88 to 76 mg/kg which we have observed in rats(2) after this treatment for 5 days with iodine.

Experiments with radioactive iodine demonstrated that ANTU inhibits the deposition of iodine by the thyroid but does not affect the uptake of iodine by other tissues. Much higher doses of ANTU were required in guinea pigs than in rats to demonstrate this effect which is in agreement with the greater resistance of guinea pigs toward the acute toxic effects of ANTU.

Summary. Total, inorganic, and organic iodine of the tissues of normal and ANTU-poisoned rats was measured. ANTU decreased the thyroid iodine of rats and guinea pigs and caused a small increase in the total iodine of blood and lung when acutely toxic doses of the rodenticide were administered. Feeding a high iodine diet for 5 days caused a marked increase in the total iodine of tissues from normal and thyroidectomized rats. Since pre-treatment of thyroidectomized rats with iodine does not afford protection against ANTU the increase in thyroid iodine of normal iodine-fed rats appears to be involved directly in the prophylactic effect of iodide against ANTU poisoning.

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Progonadotrophic Property of Dilute Antigonadotrophic Serums.* (17780)

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Augmentation of the activity of pituitary gonadotrophic preparations by the blood serum of animals which have been protractedly treated with pituitary gonadotrophins has been observed to occur in the test animal when the 2 substances are simultaneously but separately injected. Ordinarily this property appears in the early stage of the treatment and is eventually replaced by an inhibitory one. It has also been noted that an antiserum may prove to be inhibitory when tested against the pituitary gonadotrophic extract of one species but augmentative with those of one or more other species. Finally, a third phenomenon is recognized wherein some strongly inhibitory serums can be made to exhibit augmentative activity when administered in sufficiently small amounts. With regard to this phenomenon references are to be found in articles by Noble *et al.*(1), Thompson(2), and Katzman, Wade, and Doisy(3).

The present report concerns itself with this latter property as it has been observed under the conditions employed in this laboratory.

Materials and Methods. For the production of the antigenadotrophic substance young sexually mature rabbits and 60-90-day-old male rats of the Sprague-Dawley strain were used. These animals were injected daily subcutaneously with 250-500 mg[‡] of crude

sheep pituitary extract prepared according to the method of McShan and Meyer(4). At intervals during the injection period these animals were bled from the heart and the serum stored frozen until assays were performed.

Assays were carried out with 21-day-old Sprague-Dawley female rats weighing 40-50 g. Uterine weights were used as indices of activity. A total dose of 5 mg of sheep pituitary extract in saline was given in a series of 6 injections over a period of 3 days with autopsy performed on the morning of the fifth day. The antigenadotrophin was injected at a separate site from the gonadotrophin but concomitantly with it. The amount of antiserum given was varied to suit the purpose of the particular experiment being carried out. However, complete inhibition of 5 mg of pituitary extract was usually obtained with a total dose of .3 ml of antiserum administered in 6 separate injections.

Results and Discussion. As one approach to the study of the progonadotrophic property antigenadotrophic serums were diluted and each dilution assayed separately against a constant amount of pituitary extract. Fig. 1 illustrates the distribution of uterine weights when plotted against the dose of antigenadotrophic serum. Included in this figure are assays made of rat and rabbit blood serum and of rabbit lymph plasma. All antigenadotrophic serums and the lymph plasma were collected from rats or rabbits which had been treated with 250-500 mg of crude sheep pituitary extract daily for periods varying from 80-200 days. The lymph plasma was obtained by cannulation of the thoracic duct of immunized rabbits, and it has been shown to have much the same antigenadotrophic activity as blood serum (Ely, Meyer and McShan)(5). Some few of these serums were

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[‡]Indicates in all cases milligram equivalents of original pituitary powder used in making the preparation.

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taken from antihormone animals which had received a single injection of adrenal cortical extract previous to bleeding. Data have been presented (Ely, Meyer and McShan)(5) indicating that this treatment does not markedly alter the antihormone titer. Each point on the figure represents an average of the uterine weights from 2-5 animals with the great majority being averages of those from 3 animals. A total of 329 animals was involved in the assay of 17 different serums. The average uterine weight for 130 controls which received 5 mg of crude sheep pituitary extract only was 45 mg.

It will be noted that with decreased dosage there is an increase in uterine weights which at the lower concentrations is quite marked. Statistical analysis shows that the difference between the mean uterine weight obtained at the .005 ml dose level and the mean uterine weight of controls is highly significant. It should be pointed out, however, that a few of the serums assayed failed to exhibit augmentation at any dilution level. These data have also been included in Fig. 1.

Normal rat and rabbit serum gave no evidence of augmentation when doses ranging from 1.5 ml to .005 ml were assayed by the

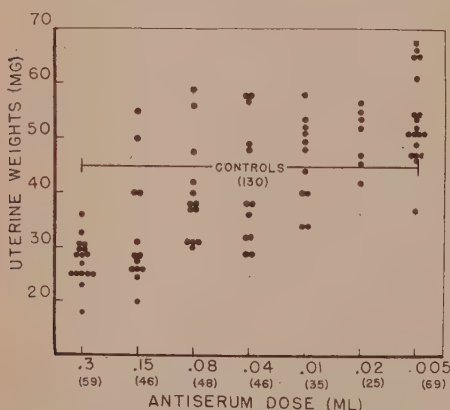


FIG. 1.

Augmentation of the effect of crude sheep pituitary extract by dilute antigonadotrophic serums of rat and rabbit blood serum and rabbit lymph plasma on the immature rat uterus. Numbers in parentheses refer to the total number of assay animals treated with a particular serum dose; each point on the graph represents the average uterine weight of from 2-5 animals assayed at a given time.

same technique as that applied to antiserum.

It should be emphasized that the capacity of antigonadotrophic serums to become augmentative upon dilution is probably a function of several factors including activity of the preparation injected, length of injection period, and the size of the immunizing dose, and that under different experimental conditions the augmentative effect might be expected to appear at different times after the initial injection and at different dilution levels from those obtained in the study reported here. Several suggestions have been advanced to explain the augmentative activity of serums of animals which have been treated with gonadotrophic materials. These have been discussed by Thompson(6), Rowlands (7), and Rowlands and Williams(8). It is evident, however, that none of these proposals has seemed entirely satisfactory. While a full discussion of the possible interpretation of the phenomenon described here with additional suggestive data is to be taken up elsewhere, it is perhaps worth mentioning that the appearance of augmentation upon dilution may have an immunological explanation. For example it is well known that in certain cases excess antigen in an antigen-antibody system results in the failure of complete precipitation of antibody and may lead to the formation of a soluble antibody-antigen complex. See Kabat and Mayer(9), and Boyd(10). Thus if the assumption is made that upon the introduction of small amounts of antibody (antigonadotrophin) into the test animal an antigen excess is created which results in the formation of soluble antigen-antibody (gonadotrophin-antigonadotrophin) complexes which slowly dissociate releasing small amounts of gonadotrophin, then the aug-

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mentative effects of very small doses of anti-gonadotrophic serum which have been shown may be explained.

Summary. It has been shown that the majority of the antigonadotrophic serums of rats and rabbits will augment the effect of a crude sheep pituitary extract upon the uterine

weight of immature female test rats when administered in sufficiently small doses. A possible explanation of this phenomenon is discussed.

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Effect of Adrenocorticotrophic Hormone (ACTH) on Serum Hyaluronidase Inhibitor in Rheumatic Fever.* (17781)

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Previous studies have shown that the "non-specific" inhibitor of hyaluronidase is elevated in acute rheumatic fever with a subsequent drop below normal levels(1). A similar rise in acute rheumatic fever was found by Grais and Glick(2). More recently Epstein *et al.*(3) failed to confirm these results claiming that no difference was observed between normal plasma and plasma obtained from patients with acute rheumatic fever. The studies of the latter investigators are open to serious question since oxalated blood was employed in these investigations. It has been demonstrated repeatedly that oxalate markedly decreases the hyaluronidase inhibitory activity of blood probably by precipitation of magnesium(4,5).

The striking effect of adrenocorticotrophic hormone (ACTH) on the course of rheumatic fever suggested that some change might be

brought about in the hyaluronidase inhibitor, under the influence of this hormone. The data to be reported were obtained on serial sera of 4 children with chronic rheumatic fever. All patients had been ill for a minimum of 7 months before treatment with ACTH, and had striking objective signs and symptoms of rheumatic activity as evidenced by elevated sedimentation rates, tachycardia, and other signs of myocarditis. They were studied on a metabolic ward for 2 weeks before treatment, during at least one month of treatment, and minimum of 2 weeks after cessation of therapy. Details of clinical and metabolic studies will be published separately. All of these patients showed marked clinical and laboratory evidence of remission of rheumatic disease during the course of therapy. Reactivation followed cessation of therapy in all patients.

Methods. Hyaluronidase inhibitor was determined as previously described by the turbidimetric method(6). This method has been shown to have a coefficient of variation of $\pm 14\%$. All sera were analyzed with and without magnesium in 2 dilutions and each determination was done in duplicate. It has been previously shown that the addition of magnesium markedly enhances inhibitor activity(5). Calculations were made in the manner previously described(6). The enzyme was a partially purified bovine testicular

* Adrenocorticotrophic Hormone (ACTH) was obtained from Armour Laboratories through the courtesy of Dr. John R. Mote.

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6. Dorfman, A., Ott, M. L., and Whitney, R., *J. Biol. Chem.*, 1948, v174, 621.

hyaluronidase which contained 960 units per mg of nitrogen. The hyaluronidase unit was arbitrary as previously defined(7). Inhibitor activity was expressed as units of hyaluronidase inhibited per ml of serum. All bloods were allowed to clot for approximately one hour and then stored at -20°C . Sedimentation rates were determined by the Wintrobe(8) method and uncorrected values are given in the graph. Eosinophil counts were determined by the method of Randolph (9).

Results. The relationship of hyaluronidase inhibitor to dosage of ACTH, sedimentation rate, and eosinophil counts for a typical patient is shown in Fig. 1. The hyaluronidase inhibitor as measured with or without added magnesium shows a marked drop upon administration of ACTH which roughly parallels the clinical course and drop in sedimentation

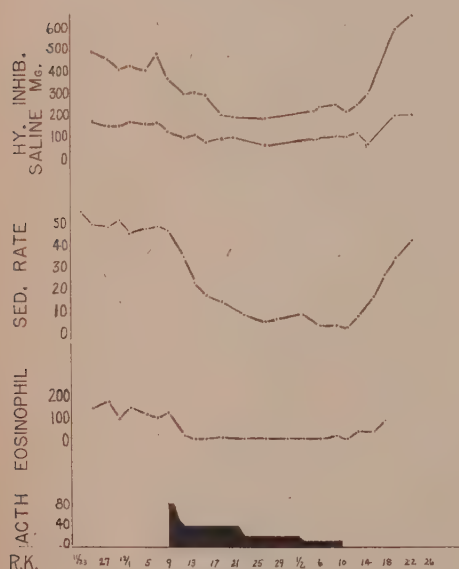


FIG. 1.

Relationship of hyaluronidase inhibitor to sedimentation rate, eosinophil count and ACTH dosage. Other patients studied showed essentially identical results.

rate. On cessation of ACTH therapy the re-appearance of evidence of clinical activity is accompanied by a rise in the hyaluronidase inhibitor. In 2 other patients, one with rheumatoid arthritis and the other with lupus erythematosus disseminata, identical results were obtained. It has been consistently found that the level of inhibitor drops below the average normal value of children of this age. A series of 14 normal children ages 9-13 gave values of 361 units with added magnesium and 128 without magnesium.

Discussion. The significance of the "non-specific" hyaluronidase inhibitor in the blood of the human is at present unknown. It seems a reasonable assumption that it is not a specific antibody, since all human bloods so far examined inhibit testicular hyaluronidase. The apparent destruction of connective tissue in rheumatic fever and related diseases, the occurrence of hyaluronic acid in connective tissue, and the production of hyaluronidase by certain strains of hemolytic streptococci have led several investigators to speculate as to the possible role of hyaluronidase in these diseases(10-12). The hyaluronidase inhibitor has been considered as a specific protective substance(13). Although it has a specific *in vitro* action there is as yet no evidence that the serum inhibitor is involved in the protection of connective tissue.

Another possible interpretation of the significance of this substance is that the level of hyaluronidase inhibitor may be a reflection of the state of the connective tissue and that the inhibitor may represent some substance which is released on destruction of connective tissue. The decrease in inhibitor under the influence of ACTH (acting by stimulation of adrenal cortical hormone secretion) may be a reflection of change of state of connective tissue brought about by adrenal hormones. The drop of the inhibitor below normal levels may indicate that the connective tissue is

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changed beyond its normal state. Such an interpretation would be consistent with the recent findings by Seifter *et al.*(14), that the alarm reaction in rabbits makes the synovial membrane less susceptible to the action of hyaluronidase. It might then be suggested that cortisone changes the character of connective tissue in such a manner that it no longer reacts to the stimuli which bring about its breakdown in the various diseases of connective tissue. An altered reactivity after ACTH therapy in response to experimental wounds has been shown to occur in rabbits

by Ragan *et al.*(15).

Summary. ACTH brings out a marked decrease in the level of the "nonspecific" hyaluronidase inhibitor in patients with rheumatic fever. This decrease parallels the drop in sedimentation rate and clinical course. It is suggested that this may be a reflection of the effect of adrenal hormones on changing the state of connective tissue so that it no longer reacts to the precipitating stimuli in the diseases of connective tissue.

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Renal Glycosuria Induced by Adrenocorticotrophic Hormone.* (17782)

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That anterior pituitary hormones will, under suitable conditions, produce temporary or permanent diabetes in the experimental animal is well recognized(1,2). Conn and his co-workers(3) have studied the temporary diabetic state which was induced in normal adult human beings by the continued administration of moderately high doses of pituitary adrenocorticotrophic hormone (ACTH) and have demonstrated that the diminished glucose tolerance and glycosuria which may follow multiple injections of ACTH are associated with diminished blood glutathione levels. Indeed, it was shown that the administration of glutathione will bring about temporary diminution of the glycosuria and hyperglycemia

induced by ACTH(4). This finding as Conn *et al.* have pointed out, is of considerable interest in relation to the studies of Lazarow(5) in which it was demonstrated that the effectiveness of alloxan as a diabetogenic agent could likewise be reduced by glutathione and other sulfhydryl-containing compounds.

In the course of studies dealing with the effect of ACTH on acute infectious diseases we have observed, in agreement with Conn *et al.*, that glycosuria could usually be induced if sufficiently large doses of ACTH were administered, and that hyperglycemia and impaired glucose tolerance generally accompany the glycosuria. Significant decreases in glutathione levels in the blood have always accompanied these evidences of altered carbohydrate metabolism. It has been suggested that one of the effects of ACTH is to bring about lowering of the "renal threshold" for glucose(3). However, the patients whose

* Aided by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service.

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2. Russell, J. A., *Physiol. Rev.*, 1938, v18, 1.

3. Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Lab. and Clin. Med.*, 1949, v34, 255.

4. Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Clin. Invest.*, 1949, v28, 775.

5. Lazarow, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, v66, 4.

responses to ACTH suggested such an effect have demonstrated abnormal glucose tolerances and usually hyperglycemia as well. The possibility exists therefore that the glycosuria was due to transitory peaks of alimentary hyperglycemia which, because of the diminished tolerance to glucose induced by ACTH, reached higher levels than usual and thus permitted the blood sugar levels to exceed otherwise normal renal thresholds.

Increased excretion of sulfur-containing compounds in the urine and feces is induced by ACTH(6). This finding, and the decrease in blood glutathione which accompanies ACTH-induced glycosuria, suggested the possibility that some of the apparent glycosuria was due to increased excretion of sulphydryl compounds. In the case to be described, apparent glycosuria occurred in the absence of evidence of impaired glucose tolerance in a patient with pneumococcal pneumonia who was being treated with ACTH. The glycosuria was accompanied by diminished blood glutathione levels, and both returned to their normal values after ACTH was withdrawn. Blood reduced glutathione was determined by the method of Woodward and Fry(7).

The patient, a 16 year old schoolboy, was admitted to the Boston City Hospital with left lower lobe type 8 pneumococcal pneumonia with bacteremia. At entry he was acutely ill with a temperature of 104°F, pulse 120 and respirations 36. The white blood count was 16,500 with 88% polymorphonuclear leucocytes and the urine was entirely negative. After an 8-hour period of observation, the patient was given 25 mg (Armour Standard) ACTH[†] per dose for 2 doses, 50 mg for 2 more, 200 mg per day for the next 2½ days, and then 175, 150, 100, 50 and 12.5 mg per day, in 4 doses, for each of 5 successive days. All injections were intramuscular. During the 12 hours after the onset of therapy the patient rapidly became afebrile, and within 18 hours he was entirely free of symptoms. By

the fourth hospital day, 3 days after the onset of therapy, the leucocyte count had dropped to 8000, the chest was clear by physical examination and virtually clear by X-ray, and the patient continued to be entirely free of symptoms. Sputum was no longer available but type 8 pneumococci were present in the throat culture.

Glycosuria developed after 24 hours of therapy with ACTH and continued until this therapy was stopped. Seven fasting blood sugar determinations made at regular intervals during the period of ACTH therapy were between 76 and 103 mg%. The urine on the third day of therapy, reduced Benedict's reagent to yellow and continued to do so as long as 200 mg per day of ACTH were administered. As the dose of ACTH was diminished the glycosuria slowly disappeared. During the second day of ACTH therapy, when the patient appeared clinically well, 6.3 g of glucose were recovered from his urine. On the third day after the onset of treatment the fasting blood sugar was 103 mg% and a glucose tolerance test after the intravenous injection of 50 g of glucose showed blood glucose levels of 160 and 87 mg% in ½ and 2 hours, respectively. The blood glutathione was 15.3 mg% on the fasting specimen and was 14.7 on the following day.

In order to determine the nature of the reducing substance, the urine was treated with washed yeast cells for 30 minutes; no reducing substances were demonstrable by Benedict's reagent after such treatment whereas prior to fermentation the reagent was reduced to a yellow color. In addition, an osazone was isolated from the urine and after recrystallization had the characteristic crystalline structure of glucosazone. The patient was seen 2 weeks after his discharge from the hospital and at this time had no glycosuria and his blood glutathione was 24.0 mg%.

Discussion. Despite the lowered blood glutathione content and evidence that the urinary excretion of sulfur-containing compounds is increased during the administration of ACTH(6), the reducing substance in the urine of a patient who received the hormone and exhibited normal glucose tolerance was primarily glucose. The methods used for

6. Kinsell, L. W., *et al.*, Proc. of the First ACTH Conference, Blakiston Co., Philadelphia, 1950, p. 70.

7. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, 1932, v97, 465.

[†] The ACTH was supplied by Dr. John R. Mote, Medical Director of Armour Laboratories.

urinary sugars do not detect minute increases in the urinary sulfhydryl content. It appears then that glycosuria may occur during the administration of ACTH with no apparent evidence of disturbed carbohydrate metabolism, but in the presence of diminished blood glutathione levels. Such a finding offers evidence that ACTH acts to depress the renal threshold, and that a temporary renal glycosuria may be induced by the hormone. It is obviously of interest to study the sulfhydryl levels in patients with true renal glycosuria. The renal threshold has been shown to be dependent upon at least 2 variables, namely, the rate of glomerular filtration of glucose, and the rate of tubular reabsorption from the glomerular filtrate(8-10). The first of these factors has been investigated and there is evidence that ACTH and cortisone act to increase glomerular filtration(1). The amount by which glomerular filtration is so increased is small, as measured by inulin clearance, and it appears unlikely that the glomerular filtration factor alone could account for the rather large amount of glycosuria in this patient. That tubular function of seemingly normal kidneys is markedly altered in the presence of large doses of ACTH or cortisone has been observed(11), and while no studies of the tubular maximal rate of absorption of glucose have been conducted as yet, it appears likely that the renal glycosuria induced in this case is due more to tubular functional changes than to alterations in the rate of glomerular filtration.

The findings in this patient also suggest that the general metabolic effect of ACTH on carbohydrate metabolism as evidenced by hyperglycemia and impaired glucose tolerance is separable, perhaps merely as a matter of degree, from the more localized effect of ACTH on renal tubular function, the latter effect

occurring at lower levels of ACTH activity before the more generalized effect appears. The kidney would appear to be peculiarly susceptible to the action of adrenocortical hormones.

It has been suggested that the effect of ACTH on carbohydrate metabolism and perhaps also on the renal threshold for glucose varies with the preparation of ACTH, and may therefore represent a factor separable from other effects of ACTH(12). Thus, Conn *et al.* have demonstrated that whereas the effect of ACTH on carbohydrate metabolism may be reversed by glutathione, the sulfhydryl compound did not affect the rate of corticoid excretion(4). Individual variations in response to the drug are, of course, to be anticipated, but there are not as yet sufficient data to decide whether the effects which have been observed are due to a contaminant or are related to the fundamental action of cortical hormones in tissue metabolism. The reproducibility of the effect and the agreement with experimental observations of the effect of adrenal cortical hormones on carbohydrate metabolism would favor the latter concept (13,14).

The possibility has been considered that the changes in glucose threshold herein described are related to the patient's infection. The patient was clinically asymptomatic at the time the observations were made. Furthermore, the disturbed glucose tolerance characteristic of severe infection was not present(15). It is of interest that disturbance of renal function observed in some patients with lobar and primary atypical pneumonias who were receiving ACTH could be reproduced in noninfected patients previously known to have normal kidney function(16), indicating that the renal functional changes were referable to the

8. Shannon, J. A., and Fisher, S., *Am. J. Physiol.*, 1938, v222, 765.

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14. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, v168, 583.

15. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams & Wilkins Co., Baltimore, 1946, p. 342.

16. Ingbar, S., Kass, E. H., and Finland, M., unpublished data.

ACTH and not to the infections. To what degree the alteration of glucose resorption and other tubular functions as well as altered carbohydrate metabolism are related to sulphydryl activity is a problem that deserves further attention.

Summary and conclusions. Glycosuria in the presence of normal blood sugar levels and normal glucose tolerance was demonstrated in a patient recovering from pneumococcal

pneumonia during treatment with ACTH. The reducing substance in the urine was shown to be glucose and the glycosuria was accompanied by lowered blood glutathione levels. These observations indicate that renal tubular function may be affected by ACTH and that sulphydryl activity may be of importance in tubular function.

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Hormone Replacement Therapy in the Aged Female—Estrogen Bioassay.* (17783)

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The practical importance of a "human unit" as opposed to a comparative animal unit in the clinical investigation and utilization of estrogen preparations is well recognized. A useful means by which such a unit may be derived lies in the comparative abilities of various forms of estrogen to produce withdrawal bleeding from the uteri of castrate women. The study presented here applies this method, described by Allen(1) to the determination of the relative effectiveness of pure alpha estradiol,[†] beta estradiol,[‡] pure estrone,[†] and estradiol benzoate[§] in causing withdrawal bleeding from the uteri of postmenopausal women.

Method of study and results. The group used for these determinations consisted of women between the ages of 60 and 86. Initially each member was given one milligram of estradiol benzoate three times per week intramuscularly and caused to bleed periodically by the withdrawal of this hormone. This manner of reactivation of the senile endometrium has been described in detail in previous publications(2,3). When a well stimulated and

TABLE I.
Typical Estrogen Bioassay.
(9 patients in each series for 3 wk).

Drug	Dosage, mg/wk	Total dosage, mg	No. showing withdrawal bleeding
Estradiol benzoate	1 × 3	9	6
Alpha estradiol	1 × 3	9	2
Estradiol benzoate	1 × 3	9	5
Estrone	1 × 3	9	2
Estradiol benzoate	1 × 3	9	2
" "	1 × 3	9	6
Beta estradiol	5 × 3	45	0
Estradiol benzoate	1 × 3	9	1

responsive endometrium had been achieved the bioassays were carried out as in Table I.

It had become evident that in order to obtain uniform results, it was necessary to interpose between each 2 of the drugs being assayed "priming cycles" of an estrogen preparation known to be a good stimulator. By previous investigation it had been found that alpha estradiol benzoate when given in dosage of 1 mg 3 times per week was capable consistently of causing withdrawal bleeding in over half of the trials. Since this comes close to a theoretical human unit or bleeding dosage of the medication, estradiol benzoate was used as a control preparation to which the estrone, alpha estradiol, and beta estradiol

1. Allen, W. M., *Southern Med. J.*, 1944, v37, 270.

* The medication used in this study was generously supplied by †The Food and Drug Administration, ‡ Organon, Inc., § Schering Corp.

2. Masters, W. H. and Allen, W. M., *J. Gerontol.*, 1948, v3, 183.

3. Masters, W. H. and Magallon, D. T., *Am. J. Obst. and Gynec.*, press.

TABLE II.
Effects of Various Dosages of Estradiol Benzoate, Alpha Estradiol, Beta Estradiol, and Estrone in Induction of Withdrawal Bleeding in Postmenopausal Patients with Adequate Priming for 3 Weeks.

Drug	No. of patients	Dosage, mg/wk	Total dosage, mg	Fraction of total No. bleeding	%
Estradiol benzoate	12	1 × 3	9	7/12	
" "	12	1 × 3	9	5/12	
" "	9	1 × 3	9	6/9	
" "	9	1 × 3	9	6/9	
Total	42	1 × 3	9	24/42	57
Estradiol benzoate	12	1 × 2	6	3/12	
" "	12	1 × 2	6	10/12	
" "	12	1 × 2	6	5/12	
Total	36	1 × 2	6	18/36	50
Alpha estradiol	9	1 × 3	9	2/9	26
Beta "	9	5 × 3	45	0	0
Estrone	12	1 × 3	9	3/12	
" "	9	1 × 3	9	2/9	
Total	21	1 × 3	9	5/21	24

TABLE III.
Treatment of Estradiol Benzoate Data Shown in Table II by the Behrens Method.

Dose	Actual No. cases bleeding	No. cases not bleeding	Behrens* No. cases bleeding	No. cases not bleeding	% bleeding
3 mg/wk	24	18	42	18	70
2 "	18	18	18	36	33½

* Behrens, *Arch. Exp. Path. Pharmac.*, 1929, v140, 237.

might be compared. The interposition of these priming cycles (estradiol benzoate 3 mg per week for 3 weeks) between each 2 of the other compounds under study also assured us that the uteri being tested remained responsive.

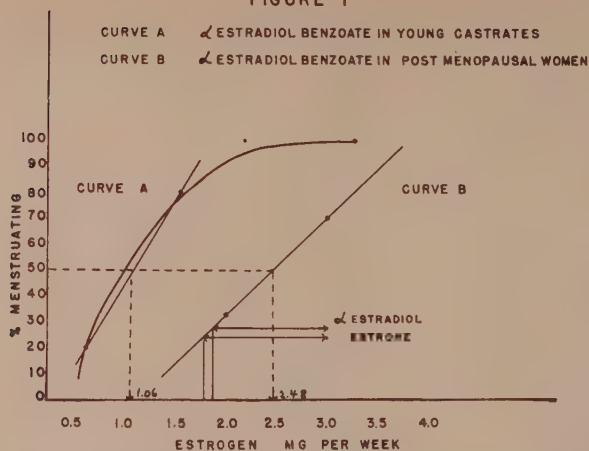
The bioassays were then carried out as shown in Table I. A 2 weeks rest period (7 days for estrogen withdrawal and 7 days for actual bleeding) was allowed between each 2 of the determinations. It will be noted that 2 priming cycles were run between the estrone and beta estradiol assays. The second priming cycle was considered necessary because of the poor response (2 out of 9 patients treated showing withdrawal bleeding) which immediately followed the comparatively ineffective estrone cycle.

Table II summarizes the data obtained on the effects of estradiol benzoate, alpha estradiol, beta estradiol, and estrone in the induction of withdrawal bleeding in postmenopausal patients with adequate priming. The term adequate priming implies that every trial

represented in Table II was preceded by a priming cycle of estradiol benzoate following which withdrawal bleeding occurred in at least one half of the cases.

A total of 78 observations were made using estradiol benzoate at 2 different dosage levels. Treatment of these results by the Behrens method (Table III) offers a way in which the data may be augmented without actually increasing the number of experiments. In applying this method, devised by its author for deducing the minimum lethal dose of certain drugs, one assumes that a single observation with a given dose was made. In this instance 42 observations were made on a dosage level of 3 mg per week with 24 bleeding, and 36 observations were made using a dosage level of 2 mg per week with 18 bleeding. The assumption, then, is made that if all patients bleeding on the smaller dose (*i.e.* 18) had been given a larger dose they would surely have bled on that dose and that all patients who failed to bleed on a given dose would surely have failed to bleed on a smaller dose.

FIGURE 1



Curve B shows the percentage of cases menstruating following the use of alpha estradiol benzoate, estrone, and alpha estradiol in postmenopausal women. Curve A shows the percentage of cases menstruating following the use of alpha estradiol benzoate in young castrates.

Table III shows the actual figures obtained on the estradiol benzoate observations and these results treated by the Behrens method. An analysis of the data in this manner shows that in a significant number of individuals given 3 mg of estradiol benzoate per week 70% might be expected to show withdrawal bleeding, and on 2 mg per week only 33 1/3% might be expected to bleed. Thus a bleeding dose or a human unit (the minimal amount of estradiol benzoate necessary to produce withdrawal bleeding in 50% of the trials) might be expected to lie between 2 and 3 mg per week.

When these values are plotted as in Fig. 1 (curve B) the dose which should induce withdrawal bleeding in 50% of the trials, the calculated bleeding dose of estradiol benzoate, is found to be 2.48 mg per week. It is interesting to note by comparison of curves A and B of Fig. 1 (from data obtained by Allen in 1944) that the theoretical dose of estradiol benzoate required in young castrates for the induction of withdrawal bleeding in 50% of the cases is 1.06 mg per week, almost 1 1/2 times less than that required in the postmenopausal or physiological castrates. One finds that the same holds true for estrone injected intramuscularly. Again, referring to Allen's

observations in young castrates, it is found that 3 mg of estrone per week was adequate to induce withdrawal bleeding in 50% of those tested. In postmenopausal women 3 mg of estrone per week caused withdrawal bleeding in only 24% of the cases. When this data is projected on the estradiol benzoate curve B in Fig. 1 it may be predicted that the biological activity of 3 mg per week of estrone is equal approximately to that of 1.74 mg per week of estradiol benzoate. In a like manner, the activity of 3 mg of estradiol would be comparable to that of 1.8 mg of estradiol benzoate.

Beta estradiol has been considered for some time to be relatively inactive. In these studies a dosage of 15 mg per week for a period of 3 weeks was given to 9 adequately primed women as shown in Table II. None of the 9 demonstrated withdrawal bleeding. The complete ineffectiveness of beta estradiol in a total dosage of 45 mg as far as stimulation of the endometrium is concerned is supported by the fact that the estradiol benzoate priming cycle which immediately followed caused only one out of 9 patients to bleed by withdrawal.

Vaginal smears taken daily and stained by

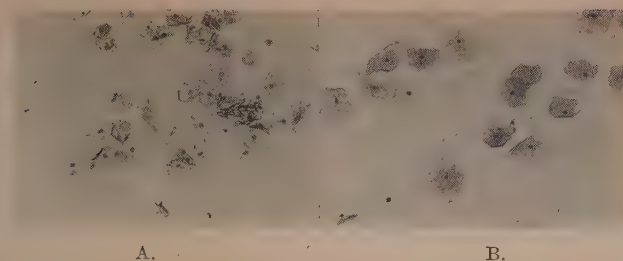


FIG. 2.

A demonstrates a vaginal smear composed of relatively unstimulated epithelial cells obtained after 17 days of beta estradiol therapy (5 mg given 3 times per week). Note the small cells with vesicular nuclei and the absence of cornified cells. B shows a vaginal smear made up of well stimulated epithelial cells taken from the same individual after 17 days of estradiol benzoate therapy (1 mg given 3 times per week). Note the large, clear, flat cells with small pyknotic nuclei. About half of the cells in this smear show precornification or full cornification.

TABLE IV.
Effects of Estradiol Benzoate and Alpha Estradiol in Induction of Withdrawal Bleeding in Postmenopausal Patients Without Adequate Priming. Dosage 1 mg $\times$ 3 weekly for 3 weeks. Total dosage, 9 mg.

Drug	Preceding cycle	No. patients	Fraction of total No. bleeding	%
Estradiol benzoate	Alpha estradiol	9	5/9	
" "	Estrone	9	2/9	
" "	Beta estradiol	9	1/9	
Total		27	8/27	29.6
Alpha estradiol	Estrone	12	1/12	8.3

the Shorr technic showed that the vaginal epithelium remained almost totally unstimulated. Fig. 2 compares the vaginal smear taken from a patient on the seventeenth day of a 21 day beta estradiol cycle (A) with a smear taken from the same individual on the seventeenth day of a 21 day estradiol benzoate cycle (B). Although the variation in color is not clearly demonstrated in the black and white illustrations, the difference in morphology of the vaginal epithelial cells after use of the 2 compounds is quite evident.

Fig. 2 (A) (beta estradiol) shows a relatively unstimulated vaginal smear consisting of small cells with comparatively large vesicular nuclei. No cornified cells are present. By contrast, Fig. 2 (B) shows a smear which demonstrates the characteristics of good estrogen stimulation. The cells are large, flat, and have very small pyknotic nuclei. About

half of the cells in this photomicrograph are "pre-cornified" or "fully cornified", the color varying from a light purple to a clear deep pink.

It was noted early in this study that the amount of recent priming to which the uteri had been subjected played a definite part in the degree of response that might be expected from the subsequent use of various preparations. Table IV shows the diminished effects of estradiol benzoate and alpha estradiol in the induction of withdrawal bleeding when priming was not adequate. Estradiol benzoate is little more than half (53.6%) and alpha estradiol about one third (31.8%) as effective in poorly primed uteri.

A method such as that described above by which the relative potencies in the human female of the various forms of estrogen may be determined somewhat accurately finds practical importance in the following ways. First, it serves to pick out the more potent pure

4. Shorr, E., *Science*, 1941, v94, 545.

5. Shorr, E., *J. Mt. Sinai Hosp.*, 1945, v12, 667.

or relatively pure drugs that may be used therapeutically not on the basis of results in the animal, but rather on the basis of response actually seen in the human uterus. Secondly, such observations may provide a fairly reliable index for clinicians in their use of mixed estrogens. It becomes apparent that such drugs may have a wide range of biologic potency milligram for milligram depending entirely upon content of the more active estrogenic substances. In addition, a rational basis is provided for establishing therapeutically effective dosage levels of the various estrogenic preparations and the modification of these levels under certain circumstances, depending upon whether one is deal-

ing with recently or remotely stimulated uteri.

Summary. Estradiol benzoate (as a standard), alpha estradiol, estrone, and beta estradiol preparations were bioassayed using withdrawal bleeding from previously stimulated uteri of postmenopausal women as an assay end point. Alpha estradiol and estrone were equally effective in causing withdrawal bleeding, and each was about two-thirds as potent as estradiol benzoate. Beta estradiol was found to be altogether ineffective in a total dosage of 45 mg given over a 3 week period.

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Effect of Antrum Transplantation on Gastric Secretion in Experimental Animals.* (17784)

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In previous studies from this laboratory, the profound reduction in gastric secretion of Pavlov pouch dogs produced by removal of the antrum of the stomach has been recorded (1). In the present experiments, quantitative collections of gastric juice from Pavlov and Heidenhain pouch dogs and from animals with totally isolated stomach preparations have been made for control periods lasting several weeks. The antrum of the stomach has then been separated from the fundus, using great care not to interfere with its blood supply, and subsequently transplanted as a diverticulum into the duodenum and into the colon. The results have demonstrated quite conclusively that when the antrum is excluded from the gastro-intestinal

tract so that it does not come in contact with food, it exerts little or no effect on gastric secretion. When it is transplanted into the duodenum, a very profound augmentation in gastric secretion results, and this is even more marked when the antrum is transplanted into the colon.

(1) *The Effect of Exteriorization of the Antrum of the Stomach on Gastric Secretion in Pavlov Pouch Dogs.* Pavlov pouches were prepared in 3 normal dogs and quantitative collections of the gastric secretion from the isolated pouches were made for control periods of several weeks by means of the tight-fitting nylon plastic cannulas previously described (2). The antrum of the stomach was then carefully separated from the fundus and the pylorus transected. The fundus was anastomosed to the side of the second portion of the duodenum. The proximal end of the antrum was infolded and closed and the pyloric portion brought to the surface as a fistula. Care

* This work was aided by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

1. Woodward, E. R., Bigelow, R. R., and Dragstedt, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, v68, 473.

2. Dragstedt, L. R., Haymond, H. E., and Ellis, J. C. *Surg., Gynec., and Obst.*, 1933, v56, 799.

was taken not to interfere with the blood supply to the antrum. In all three animals, exteriorization of the antrum in this fashion produced a marked reduction in the secretion of gastric juice from the Pavlov pouch comparable to that secured by antrum resection. In Dog 1, the average 24 hour output of hydrochloric acid decreased from 22 to 15 milliequivalents; in Dog 2 from 73 to 17 milliequivalents; and in Dog 3 from 51 to 18 milliequivalents in a 24-hour period.

(2) *Effect of Transplantation of the Antrum into the Duodenum on Gastric Secretion in Dogs Provided with A Vagus Denervated Total Isolated Stomach.* Total isolated stomach pouches with the vagus nerves divided after the method of Lim and Ivy(3) were prepared in 4 dogs. Quantitative collections of gastric juice from the isolated stomach were secured for control periods of 2 to 3 weeks. The antrum was then excised from the isolated stomach and transplanted as a diverticulum into the side of the duodenum, taking care not to interfere with its blood supply. In all cases, the transplantation of the antrum into the duodenum produced a marked augmentation of gastric secretion by the isolated fundus. In Dog 1, the average 24 hour output of hydrochloric acid by the isolated stomach increased from 2 to 77 milliequivalents; in Dog 2 from 5 to 51 milliequivalents; in Dog 3 from 7 to 120 milliequivalents; and in Dog 4 from 9 to 45 milliequivalents. It is thus evident that the antrum has little or no effect on gastric secretion when it is present in the isolated stomach and is subjected only to the contact with gastric juice. On the other hand, marked augmentation of secretion was produced when this antral mucosa came in contact with the duodenal content.

(3) *Effect of Transplantation of the Antrum of the Stomach into the Colon on Gastric Secretion in Pavlov Pouch Dogs.* In 3 animals Pavlov pouches were prepared and the control secretion determined. The antrum was then exteriorized as in the experiments described in paragraph one. After a control period, the exteriorized antrum was then transplanted

into the middle of the transverse colon as a diverticulum and its effect on gastric secretion determined. In Dog 1, the average 24 hour output of hydrochloric acid from the Pavlov pouch under control conditions was 22 milliequivalents per 24 hours. With the antrum exteriorized, the average output fell to 15 milliequivalents per day. When the antrum was transplanted into the colon, the output of hydrochloric acid by the Pavlov pouch increased to 56 milliequivalents per day. In Dog 2, the average output of hydrochloric acid from the Pavlov pouch during the control period was 73 milliequivalents per day. When the antrum was exteriorized, the output fell to 17 milliequivalents per day. When the antrum was transplanted into the colon, the average secretion increased to 96 milliequivalents per day. In Dog 3, the average 24 hour output of hydrochloric acid from the Pavlov pouch during the control period was 51 milliequivalents per day. When the antrum was exteriorized, this fell to 18 milliequivalents, and when the antrum was subsequently transplanted into the colon, it increased to 120 milliequivalents.

(4) *Effect of Transplantation of the Antrum of the Stomach into the Colon on Gastric Secretion in Total Pouch Dogs.* Four dogs were prepared with totally isolated stomach pouches with the vagus nerves divided. After a control period of collection of gastric secretion, the antrum was separated from the isolated pouch and transplanted into the middle of the transverse colon as a diverticulum. In Dog 1, the average 24 hour output of hydrochloric acid from the isolated stomach during the control period was 20 milliequivalents, and this increased to 135 milliequivalents when the antrum was transplanted into the colon. In Dog 2, the average output of hydrochloric acid during the control period was 5 milliequivalents per day, and this increased to 75 milliequivalents when the antrum was transplanted into the colon. In Dog 3, the average output of hydrochloric acid during the control period was 13 milliequivalents per day, and this increased to 143 milliequivalents when the antrum was transplanted into the colon. In Dog 4, the average output of hydrochloric acid during the control period was 75

3. Lim, R. K. S., Ivy, A. C. and McCarthy, J. E. *Quart. J. Exp. Physiol.*, 1925, v15, 13.

milliequivalents per day, and this decreased to 19 milliequivalents when the antrum was exteriorized. When subsequently the antrum was transplanted into the duodenum, the average output of hydrochloric acid increased to 65 milliequivalents per day.

Conclusions. 1. Exteriorization of the antrum of the stomach produces a marked decrease in the secretion of Pavlov pouch dogs comparable to that secured by excision of the antrum. When this exteriorized antrum is subsequently transplanted into the duodenum

or into the colon, a marked stimulation of gastric secretion is produced.

2. Transplantation of the antrum of dogs with totally isolated stomach pouches into the duodenum or into the colon produces a marked increase in gastric secretion. These findings support the view that the antrum mucosa is a specific internal secreting organ distinct in function from the mucous membrane of the stomach or duodenum.

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Effect of Cortisone* on Experimental Fractures in the Rabbit.[†] (17785)

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It has been observed that wounds of patients treated with adrenocorticotrophic hormone (ACTH) fail at times to heal normally(1). These observations prompted an experimental evaluation of the action of cortisone on standard skin defects induced in rabbits' ears. It was established that cortisone caused a striking depression of all elements of connective tissue, including formation of new blood vessels, and produced evidence of hyperadrenalism as manifested by depression of circulating eosinophiles and mild hyperglycemia(2). In the light of these

studies, it seemed of interest to observe the effect of this adrenal steroid on other mesenchymal tissues. Consequently the effect of cortisone on callus formation was investigated and the results are reported in the following experiments.

Methods. Animals. Healthy adult laboratory rabbits of mixed breeds and sexes weighing between 2.6 and 3.2 kg were used.

Method of Fracture. Under nembutal and ether anesthesia the right femur was fractured by a sharp blow with a mallet in the midshaft without breaking the skin.

Methods of Pathological Study. Animals were sacrificed with nembutal or the intracardiac injection of air. The injured extremities were removed *in toto*, frozen, and then split through the fracture site with a band-saw. Both longitudinal and transverse sections were taken through comparable areas of the fracture site. Sections of liver and adrenals were also made. Tissues were fixed in 80% alcohol, chilled acetone and alcohol-formalin, and were imbedded in paraffin. Sections of the fracture site were stained with the following technics: Hematoxylin and chlorazol fast pink B; Tol-

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uidine Blue O—1:1000 according to Lillie(3), Hale(4), Masson Trichrome; Laidlaw Silver, Feulgen(5), Feulgen without hydrolysis, periodic acid Schiff reaction(6), periodic acid Schiff reaction after diastase(3), alkaline phosphatase(4), and alkaline phosphatase control. Sections of the liver were stained with hematoxylin and chlorazol fast pink B and the periodic acid Schiff reaction after and without diastase. Sections of the adrenals were stained with hematoxylin and chlorazol fast pink B.

Experimental. Paired rabbits were used. One rabbit of each pair was pretreated with 12.5 mg of cortisone acetate intramuscularly twice daily for 3 days before fracture and thereafter until the time of sacrifice. The other animal of each pair was used as a control and was otherwise treated exactly the same as the rabbit receiving cortisone. Following the pretreatment period in 8 pairs of rabbits, fractures were produced according to the technic described. Treatment following this procedure was identical with the preoperative period. No effort was made to splint or otherwise limit motion of the fractured leg. Despite the injury the rabbits ate and drank well and appeared healthy. One pair of animals was sacrificed daily for 8 days following fracture. Autopsies failed to reveal a significant gross difference between the treated rabbits and controls except for the fracture site and the liver.

On gross examination, the reparative process was delayed in the cortisone-treated rabbits. At the second post-fracture day, the control animals showed signs of resolution of muscle hematoma but these remained jelly-like in the animals receiving cortisone. By the fifth post-fracture day, a significant zone of healing was grossly apparent in the control while at the fracture site of the treated animals there was only minimal healing between the ends of fractured bone. In the con-

trol animals, the zone of healing tissue became progressively larger over the 8-day period. However, the thin band of healing tissue seen on the fourth day in the cortisone-treated rabbit was not appreciably larger on the eighth day.

On *microscopic* section, the repair process was seen to pursue the following pattern: By the second post-fracture day, early fibroplasia could be seen and for the first 3 or 4 days there was a local reaction of edema, inflammatory infiltration and tissue autolysis. During this period of time there was little difference between the control and the cortisone-treated rabbits. Beginning with the fourth or fifth day, however, the difference between the 2 groups was striking. In the *control animals*, well-defined areas of new cartilage were seen near the old bone surrounded by a wide margin of granulation tissue which encompassed some muscle fibers. By the eighth day (Fig. 1a), well-defined soft bone trabeculae were present in association with the cartilage and the granulation tissue. The calcium of the engulfed muscle bundles decreased as the calcified trabeculae appeared. In the *cortisone-treated rabbits* (Fig. 1b), there was a retardation of all phases of healing after the fourth day. Metachromasia was decreased and the intercellular matrix appeared quite irregular in structure. Fibroblasts showed rounded forms and bizarre arrangements of nuclear chromatin. The cartilage which was seen on the eighth day in the treated animal had small lacunae, sparse perilacunar reticulum fibers, only irregular metachromasia of the matrix, minimal and irregular alkaline phosphatase content and only sparse small glycogen granules in the cytoplasm. No bone trabeculation was seen in the treated rabbits within the experimental period. In essence, up to the fourth post-fracture day, the control and cortisone-treated rabbits exhibited little difference in the reparative process. After the fourth day at about which time new blood vessels began to appear, there was a marked retardation of repair in the cortisone-treated group.

The effect on certain other organs was studied. The gross appearance of the liver of the cortisone-treated rabbits differed from

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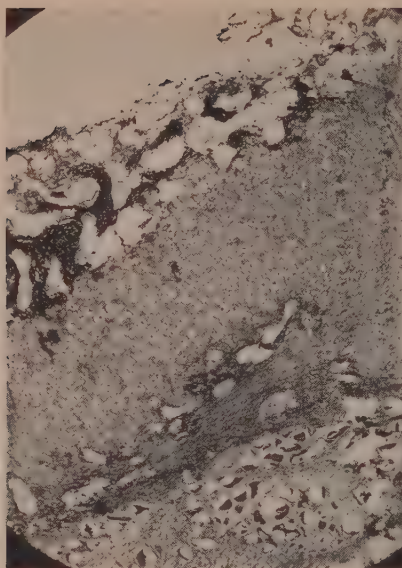


Fig. 1a. Section through fracture site, 8th post-fracture day, control rabbit, 38 X, hematoxylin-chlorazol fast pink B. At the top, below the margin of injury, is a dense area of fibroplasia. There is a wide area of well-differentiated cartilage below that and at the bottom osteoid with bone trabeculation is noted.



Fig. 1b. Section through comparable fracture site, 8th post-fracture day, cortisone-treated rabbit, 38 X, hematoxylin-chlorazol fast pink B. The entire healing zone is represented by the relatively narrow area on the right. No clearly defined cartilage and no osteoid can be seen.

the controls. From the first day of sacrifice (after 4 days of cortisone), the liver of the treated animal was larger, firmer and paler than the control, bled less and on cut section showed disturbance of the architecture by infiltration of a pale yellow substance. On the eighth day following fracture, the liver of the control animal weighed 85 g while that of the treated rabbit weighed 175 g. Histologically, the increase in weight and change in gross appearance were shown to be due, at least in part, to a marked deposition of glycogen (Fig. 2). There was no other significant histological difference noted between the livers of the two groups. The adrenals of the control and treated animals appeared grossly the same and histologically no distinct differences could be seen.

Discussion. The present studies confirm and extend previous observations on the inhibitory effect of cortisone on the response of the connective tissues to trauma. By means of serial daily observations, a histological fea-

ture of these experiments became evident which may help to clarify the mechanism involved. Up to the fourth post-fracture day, the inflammatory and reparative responses of the connective tissue were practically the same in both control and cortisone-treated animals. However, at about that time, new vessels began to penetrate the area of injury in both groups, but in the control animals the number of new vessels appeared to be greater. Following the ingrowth of new vessels in the control animals, reconstruction and differentiation of tissue continued rapidly, but in the cortisone-treated animals the reparative process remained sluggish with relatively little tissue differentiation. This difference may be ascribed to one of two possible situations: first, that in the cortisone-treated animals, the blood supply was inadequate due to a dearth of new blood vessels with inadequate nutrition of tissue; or secondly, that through the few new blood vessels which did penetrate the area, a material, perhaps cortisone itself, was



Fig. 2a. Liver, 8-day cortisone-treated rabbit, 78 X, hematoxylin-chlorazol fast pink B. Note the enlargement of the hepatic cells and vacuolization of the cytoplasm.

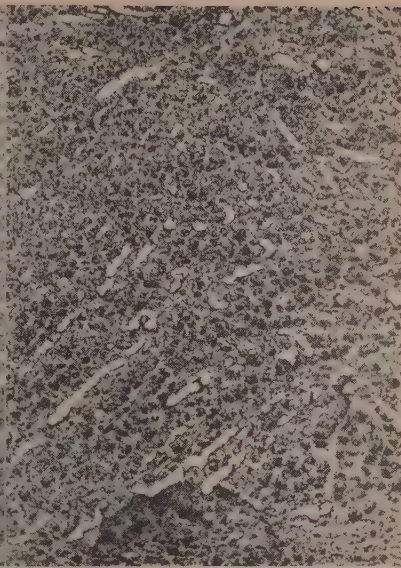


Fig. 2b. Liver, same cortisone-treated rabbit, 78 X, periodic acid Schiff stain. The cytoplasmic enlargement is seen here to be due largely to glycogen infiltration.

transported which inhibited growth and development of connective tissue. Although our data do not permit a definite conclusion as to the mechanism involved, the latter view receives some support from the observations of Baker(7), who demonstrated an inhibitory effect of cortisone applied locally to open wounds.

Summary. 1. Sixteen rabbits were subjected to fractures of the femur. Eight of these were under treatment with cortisone.

2. One control and one treated animal were sacrificed serially for eight days following fracture.

3. Gross healing of the fracture and absorption of the hematoma were greatly delayed in

the animals receiving cortisone.

4. Histologically, the chief differences were seen following the fourth post-fracture day when retardation of all phases of healing were noted in the cortisone-treated animals. At the fourth day, new blood vessels began to appear in both control and cortisone-treated rabbits, but were more numerous in the control animals.

5. Failure of connective tissue regeneration following the fourth post-fracture day in the cortisone-treated rabbits may have been the result of poor tissue nutrition due to an inadequate blood supply or may have been due to the action of a circulating material possibly cortisone itself, brought to the area through new vessels.

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Acid Phosphatase Activity in the Intestinal Wall in Experimental Amebic Colitis.* (17786)

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Acid phosphatase activity has been demonstrated in *Endamoeba histolytica* of human origin grown in artificial culture media or obtained by rectal aspiration from an experimentally infected animal(1). The present report describes the results obtained when tissue sections of amebic lesions were submitted to the acid phosphatase test.

Materials and methods. Amebic colitis was experimentally produced in guinea pigs by intra-ileal inoculation with large numbers (over 200,000) of amebic trophozoites (Tulane strain XXII) grown in Balamuth's medium(2). Four to 7 days after inoculation the animals were sacrificed and portions of cecum with extensive amebic lesions were selected for study. Gomori's histochemical technic for the demonstration of acid phosphatase in tissue(3) was used throughout the experiment. Duplicate sections submitted to identical procedure except for the absence of the glycerophosphate substrate from the incubating solution, served as controls. All sections were incubated overnight.

Results. Very heavy precipitate occurred in and around the areas of amebic invasion, (Fig. 1), revealing that intense acid phosphatase activity was present in those regions. Isolated amebae at some distance from active ulcers were also strongly positive, while the surrounding host tissues did not show any demonstrable enzymatic activity. Foci of black precipitate which did not correspond to

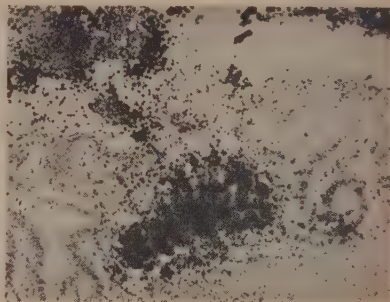


FIG. 1.

Flask shaped amebic ulcer showing heavy lead sulfide precipitate in the crater of the ulcer, and on the exudate covering the ulcer and adjacent mucosa. The host tissues surrounding the ulcer are negative. Isolated amebae in a nearby gland and in the exudate are strongly positive. Minute foci of phosphatase activity which do not correspond to amebae are also seen. The section was weakly counterstained with H & E to provide an adequate background. 100 X.

the amebae themselves were also observed, but always in, or near, active amebic colonies. It is not known, yet, what these foci of acid phosphatase activity represent, but judging from their size, shape and location, they might be either fragments of disintegrating amebic protoplasm or small microorganisms that may be present in the amebic lesions. It is worthy of emphasis, however, that in the areas not affected by the amebae, no such foci of phosphatase activity were evident.

Since all the controls were negative, it can be concluded that the precipitate represented acid phosphatase activity. The unusually strong reaction at the sites of greatest amebic invasion, as well as in the isolated amebae themselves, confirms the original impression(1) that *E. histolytica* has a high concentration of acid phosphatase.

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Alveolar Air, Respiratory Dead Space, and the "Ventilation Index." (17787)

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The purpose of this paper is to report an experimental result of unexpected simplicity obtained in the course of an attempt to devise tests for respiratory impairment. The ideas that led to this finding were somewhat as follows: The search for tests of adequacy of ventilation reduces essentially to the attempt to relate measurable respiratory characteristics to a more or less abstract system of unknown "alveolar" partial pressures and respiratory dead space, and to relate this, in turn, to the composition and distribution of blood entering and leaving the vessels of the lungs. A dilemma immediately arises, for the unknown quantities are interdependent; one way or another, one is always faced with the choice between alveolar partial pressures obtained by assuming a value for the respiratory dead space, and the converse. It is true that methods have been developed for the more direct measurement of both alveolar partial pressures and dead space, but as these are open to theoretical and practical objections, especially when they are used in conjunction with experiments involving muscular exercise, it is desirable to circumvent them. One is thrown back on the dilemma, to which such partial solutions are available as that offered by Krogh and Lindhard (1) who advocated the calculation of alveolar partial pressures from those of whole expired air by means of the Bohr equation, with the adoption of a value for the respiratory dead space such as that obtained for a limited number of normal persons in the very careful measurements of Krogh and Lindhard. The relevance of this is, however, open to question, since in those measurements an inspiratory pause was intro-

duced to ensure complete intrapulmonary mixing.

The difficulty is expressed algebraically by 2 equations which can be derived very simply from the Bohr (2) equations:

$$V_t = \frac{qO [1 - (1 - Q) \cdot fO]}{m} \cdot \frac{1}{fO - fO''} + V_d \quad (1)$$

$$V_t = \frac{qC}{m} \cdot \frac{1}{fC''} + V_d \quad (2)$$

where V_t is tidal volume; m is number of breaths per minute; qO and qC are oxygen and carbon dioxide turnover per minute, respectively; fO is the fraction of oxygen in the inspired gas; fO'' and fC'' are "effective" alveolar oxygen and carbon dioxide fractions, respectively; Q is respiratory quotient, and V_d is "effective" respiratory dead space.

The unknown quantities in these 2 equations are 3 in number: fO'' , fC'' , and V_d ; the remainder are readily measured in the laboratory. If a change in physiologic state is brought about—for example, by muscular exercise—a solution is still impossible, since one is not entitled to assume that any of the 3 unknown quantities remain unchanged. It is clear that no progress is possible along these lines without either a knowledge of the physiologic factors which might permit formulation of an additional equation, or empirical observation of the operation of those factors.

The latter approach seemed preferable, and muscular exercise the most suitable means of bringing about physiologic change. Accordingly, the measurements indicated by equations

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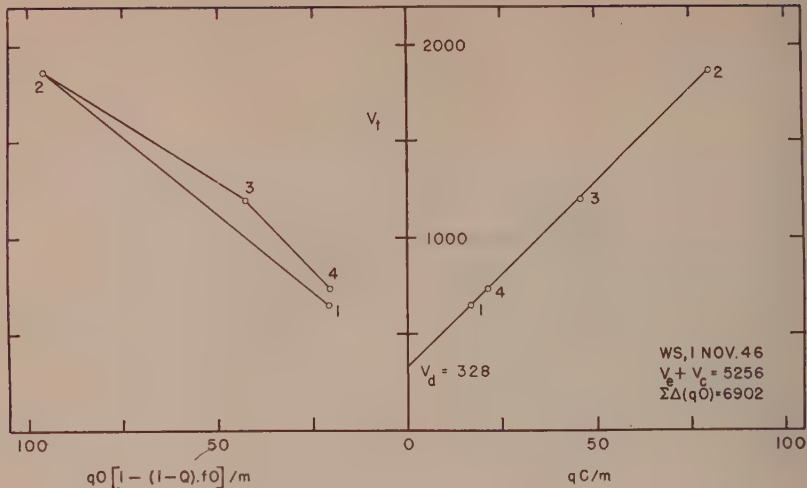


FIG. 1.

Response of tidal volume, oxygen and carbon dioxide turnover, and breathing rate to muscular exercise, plotted in accordance with text equations (1) and (2). Points marked 1 are for the person sitting at rest; 2 refers to a 2-minute exercise period (4 miles per hour, 10% grade), 3 to the first 4 minutes of recovery, 4 to the second 4 minutes. The subject's vital capacity was 5,256 cc BTPS; the extra oxygen consumption during exercise and 8 minutes of recovery was 6,902 cc BTPS. Symbols are explained in text.

(1) and (2) were performed on a group of normal persons at rest, during muscular work, and during recovery. The measurements were made by a standard open-circuit method; the exercise was done on a treadmill for a period of 2 minutes, and the recovery was followed for 2 consecutive periods of 4 minutes each. The amount of exercise varied widely and its severity was assumed to be related to the excess consumption of oxygen associated with it during the entire ten minutes of observation.

The data were examined graphically, the tidal volume being plotted against $qO [1 - (1-Q) \cdot fO] / m$ and qC/m , respectively. It was consistently observed that the values of qC/m were linear with V_t , while those of the oxygen factor usually were not (Fig. 1). The most convenient interpretation of this result is to suppose that the effective partial pressure of carbon dioxide in the alveoli and the effective respiratory dead space both remain constant in normal persons during muscular exercise of some severity and the ensuing period of recovery. In seeking any other interpretation one would be obliged to assume the linear relationship to be the result of a fortuitous

compensation of changes in alveolar carbon dioxide and respiratory dead space. Accepting the simpler explanations as the more expedient, one can at once derive from the experimental data values for (1) the effective respiratory dead space, represented by the value of the tidal volume when qC/m is extrapolated to zero; (2) the effective partial pressure of carbon dioxide in the alveoli, and (3) the effective partial pressures of oxygen in the alveoli during each of the 4 experimental periods.

The numerical data thus obtained in the entire series of 13 experiments on 9 normal persons are given in Table I, together with the vital capacities and the excess oxygen consumed during exercise and recovery. It is evident that the absolute variation in pO'' is generally rather greater, and often much greater, than that of pC'' ; this is true also of the percentage variation in $pO - pO''$, in agreement with the conclusion already drawn from inspection of the graphical data. The figures showing the constancy of pC'' provide good evidence that experiments of this kind can be of value in obtaining reliable

TABLE I.
Effective Respiratory Dead Space and Alveolar Partial Pressures Calculated From Respiratory Response to Exercise*

Subject	Vital capacity	Excess O ₂ uptake	V _d	pC''			pO''		
				Rest	Exercise	Recovery	Rest	Exercise	Recovery
Men									
JB	5145	5806	370	39.6	39.9	40.6	92.8	90.2	102.8
	4717	5180	310	36.9	37.2	38.7	94.6	91.9	101.4
	5012	2252	260	34.5	34.9	35.3	100.6	98.8	104.1
	4855	1674	280	33.2	33.1	35.0	98.8	100.2	97.2
JH	4917	1668	300	35.9	35.5	37.8	99.5	99.8	96.2
	5098	4766	280	35.1	34.9	35.3	99.8	99.8	95.8
	5265	6902	330	36.0	35.7	36.0	102.6	96.3	105.4
	6449	5640	295	35.3	34.7	33.9	98.8	100.2	108.3
H	6268	1956	330	34.0	33.4	33.7	103.7	101.9	108.0
	3737	3872	195	34.3	33.8	34.8	103.3	101.8	103.6
						34.6	103.9	106.3	106.5
Women									
R	3380	1958	205	29.6	29.6	29.3	107.5	106.5	108.0
L	3078	2370	180	30.0	29.8	30.1	105.5	106.5	109.0
P	3395	3522	155	28.3	28.1	30.4	110.5	110.5	109.5
						27.8	110.5	110.5	117.0

* Vital capacity and excess oxygen uptake in ml BTPS. Excess oxygen uptake is amount consumed in 2 min. exercise and 8 min. recovery, minus resting consumption over 10 minutes. V_a is effective respiratory dead space obtained by plotting qC/m against tidal volume. pC'' and pO'' are effective alveolar partial pressures (in mm of mercury) of carbon dioxide and oxygen, respectively, calculated from the experimental data by means of text equations (1) and (2). The 4 figures in each case correspond to rest, exercise, and the two 4-minute recovery periods. Values of effective dead space have not been corrected for dead space of face mask.

information concerning the effective partial pressures in the alveoli and the effective respiratory dead space. Such information is perhaps to be considered superior to that gained by other methods, in respect both to technical simplicity and to lack of dependence on dubious assumptions. The effective alveolar partial pressures are related in a clearly defined manner to accurately measurable quantities, on the one hand, and to a simple model of the respiratory process, on the other; consequently they should constitute an acceptable definition of the state of the gaseous phase at the site of respiratory interchange, with which can be compared the effectiveness of the lungs as judged by the gaseous exchanges of the blood.

The effective dead space obtained by the present method likewise has definite advantages as an index of ventilatory efficiency, since it represents a physiologically effective, rather than an anatomically defined, dead space, and can be expected to be sensitive to anomalies in distribution and mixing of the intrapulmonary gases. This is true also of the effective dead space obtained from lung clearance measurements, but such measurements are technically so demanding as to have little value in a routine laboratory. In view of the high degree of consistency of the present measurements, the fact that they can most easily be interpreted in terms of a constant dead space is of particular significance. It has been rather generally supposed that increased ventilation brings about an increase in the respiratory dead space, and even Krogh, who was inclined to oppose this view, was forced to concede that a moderate increase may occur.

The present considerations also point to what may be a valid form of ventilation index. The respiratory volume per unit of oxygen consumed has been used widely as a ventilation index in clinical circles and almost as widely criticized. Equation (1) shows that an index involving oxygen consumption would have to be calculated in the form $m(V_t - V_d)/qO_2$, instead of mV_t/qO_2 , but would then be constant only as long as the other quantities in the equation—notably the respiratory quotient and the alveolar oxygen partial pressure—remained unchanged. A valid index, as the data in Table I demonstrate, would be $m(V_t - V_d)/qC$, the *effective* respiratory volume per unit of *carbon dioxide* produced; but calculation of such an index is equivalent to a partial analysis along the lines of Table I and would be superfluous if the data permitted the complete calculation of V_d , fO'' and fC'' .

Summary. The ventilatory response of 9 normal persons to muscular exercise can be described in part by a linear relationship between tidal volume and carbon dioxide output per breath. The intercept (Fig. 1) is the effective respiratory dead space and the slope is the reciprocal of the effective alveolar carbon dioxide partial pressure. Thus, during exercise and recovery, involving large changes in oxygen consumption and tidal volume, both effective respiratory dead space and effective alveolar carbon dioxide remain remarkably constant. This circumstance makes possible the calculation of dead space and alveolar partial pressures without recourse to direct sampling methods.

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Excretion of Uric Acid and Allantoin by Rats Depleted of Liver Xanthine Oxidase.* (17788)

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It was previously shown(1) that rats maintained on a purified low protein diet lost nearly all(2) of the xanthine oxidase from the liver. The present study shows that it is impossible to deplete all of the tissues of this enzyme by a prolonged low protein regime, and that the 25% or less of original enzyme retained throughout the entire rat is sufficient to maintain normal excretions of uric acid and allantoin.

Experimental. Adult Sprague-Dawley male rats were maintained on chow to obtain the normal levels of tissue xanthine oxidase and the normal levels of uric acid and allantoin excretions. A group was then placed on the purified 8% casein diet previously described (3), but substituting sucrose for glucose.

Urines were collected from some of the rats after 11 and 28 weeks, and the corresponding rats were then sacrificed for an analysis of the tissues for xanthine oxidase(1,2). Since the effect of a low protein diet is intensified in a weanling rat, similar studies were conducted on 21 day old albino male rats. Some were analyzed as received; organs from several rats were pooled whenever necessary. Others were placed on the purified 8% casein diet and analyzed for uric acid-allantoin excretions and tissue xanthine oxidase after 4 or 8 weeks. Uric acid and allantoin excretions were also determined in adult Sprague-Dawley male rats receiving the carcinogenic dye, p-dimethylaminoazobenzene, since the feeding of this dye was found to decrease the liver xanthine oxidase activity. Three diets of

varying protein content (8, 12 and 21%) were fed with and without the addition of 0.06% p-dimethylaminoazobenzene. The 12% casein diet was similar to the 5% corn oil diet described by Kline, *et al.*(4), and the others varied only in the casein and glucose contents. These diets were fed for 8 to 9 weeks before urine samples were collected and the rats were sacrificed for tissue analyses. Urine samples were collected over several hours, either freely or after an oral dose of water. The urines were frozen and analyzed within a few days for uric acid, allantoin and creatinine; the uric acid and allantoin excretions were expressed as a ratio of the creatinine excreted(5). The creatinine excretion in mg per 100 g body weight per hour was also calculated and found to be unaltered by the dietary procedures.

Results. The results of the low-protein feeding experiments are shown in Table I. The adult control rats had somewhat lower tissue xanthine oxidase activities than previously found for rats maintained on chow. The purified low-protein diet had a major effect only on the liver enzyme; the small intestine also lost some activity, but the other tissues retained their xanthine oxidase. The uric acid and allantoin excretions were not affected by the loss of the liver xanthine oxidase.

Weanling rats fed purified 8% casein diet lost liver xanthine oxidase rapidly and nearly completely; the small intestine was affected similarly. Kidney was devoid of measurable amounts of enzyme at weaning and never acquired any during the dietary period. Spleen appeared to increase its xanthine oxidase activity during the dietary regime, but the changes were relatively small in light of the large individual variations found. Weanling rats fed the diet for 8 weeks represented a

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TABLE I.
Effect of Feeding a Purified 8% Casein Diet on Xanthine Oxidase Activity of Rat Tissues and Excretion of Uric Acid and Allantoin by the Rat.

	No. of rats	Body wt, g		Weeks on diet	Xanthine Oxidase Activity (Mean $\pm$ S.E.)					Uric Acid Creatinine, mg/mg	Allantoin Creatinine, mg/mg	Creatinine Excr. mg/100 g body wt/hr
		Start	End		Liver	Kidney	Small intest.	Lung	Spleen			
Adults	30*	289	—	Chow	1010 $\pm$ 55	156 $\pm$ 12	139 $\pm$ 61	242 $\pm$ 23	400 $\pm$ 49	.34 $\pm$.01	4.42 $\pm$.34	.123 $\pm$.009
	9	197	271	11	21 $\pm$ 21	184 $\pm$ 26	110 $\pm$ 25	270 $\pm$ 24	606 $\pm$ 81	.29 $\pm$.04	3.05 $\pm$.18	.131 $\pm$.007
	6	278	296	28	26 $\pm$ 9	313 $\pm$ 24	98 $\pm$ 43	358 $\pm$ 21	713 $\pm$ 78	.36 $\pm$.04	5.35 $\pm$.96	.119 $\pm$.009
Weanlings	12	35	—	0	662 $\pm$ 63	0 $\pm$ 0	493 $\pm$ 26	263 $\pm$ 15	205 $\pm$ 48	.49 $\pm$.09	9.86 $\pm$ 1.09	.103 $\pm$.016
	6	32	46	4	0 $\pm$ 0	0 $\pm$ 0	45 $\pm$ 20	189 $\pm$ 20	248 $\pm$ 60	.40 $\pm$.08	5.31 $\pm$.98	.131 $\pm$.020
	7	34	51	8	0 $\pm$ 0	0 $\pm$ 0	44 $\pm$ 21	136 $\pm$ 13	360 $\pm$ 49	.45 $\pm$.04	5.18 $\pm$.22	.102 $\pm$.025

* Uric acid and allantoin excretions determined on 30 rats maintained on chow; tissue xanthine oxidase determined on 12 rats.

TABLE II.
Effect of Feeding p-Dimethylaminobenzene On Tissue Xanthine Oxidase Activity and Excretion of Uric Acid and Allantoin By Rats.

Diet	Body wt, g		Xanthine Oxidase Activity (Mean $\pm$ S.E.)					Uric Acid Creatinine, mg/mg	Allantoin Creatinine, mg/mg	Creatinine Excr. mg/100 g body wt/hr
	Start	End	Liver	Kidney	Lung	Spleen	Intestine			
21% Protein	193	356	926 $\pm$ 42	222 $\pm$ 20	295 $\pm$ 24	461 $\pm$ 41	82 $\pm$ 36	.424 $\pm$.063	3.58 $\pm$.45	.132 $\pm$.014
" + Dye	188	324	768 $\pm$ 117	65 $\pm$ 36	366 $\pm$ 46	610 $\pm$ 98	145 $\pm$ 69	.422 $\pm$.031	3.82 $\pm$.35	.098 $\pm$.013
12% Protein	181	324	413 $\pm$ 87	156 $\pm$ 18	261 $\pm$ 42	368 $\pm$ 46	15 $\pm$ 15	.320 $\pm$.017	3.32 $\pm$.32	.132 $\pm$.014
" + Dye	191	227	105 $\pm$ 25	10 $\pm$ 10	168 $\pm$ 18	481 $\pm$ 65	146 $\pm$ 56	.416 $\pm$.046	3.58 $\pm$.18	.161 $\pm$.015
8% Protein	181	233	335 $\pm$ 48	160 $\pm$ 55	350 $\pm$ 38	370 $\pm$ 57	74 $\pm$ 26	.319 $\pm$.025	4.41 $\pm$.38	.135 $\pm$.007
" + Dye	178	201	114 $\pm$ 30	7 $\pm$ 7	225 $\pm$ 36	435 $\pm$ 77	81 $\pm$ 41	.598 $\pm$.079	5.90 $\pm$ 1.95	.100 $\pm$.011

Average of 6 rats in each group.

TABLE III.
Changes in Liver Xanthine Oxidase Independently of Fat and Glycogen.

Diet	No. of rats	Body wt, g		Liver Constituents (Mean $\pm$ S.E.)		
		Start	End	Xanthine oxidase units	Fat % dry wt	Glycogen % wet wt
Chow	10	249	366	1000 ± 47	14.9 $\pm .57$	4.92 $\pm .88$
21% Casein*	11	248	365	741 ± 53	13.7 $\pm .77$	6.06 $\pm .53$
8% Casein*	11	254	307	61 ± 33	16.3 ± 1.25	6.38 ± 1.17

* As previously described(3) except that sucrose replaced glucose.

fairly exhaustive depletion of the xanthine oxidase from the rat tissues, yet the formation and excretion of uric acid and allantoin was not altered appreciably. It should be noted that livers with "zero" xanthine oxidase activity actually have 50 to 100 units of enzyme present(2), which apparently cannot be exhausted by this dietary procedure since it has always been detected by the aerobic methylene blue procedure(2). This small amount of liver enzyme, supplemented by the xanthine oxidase left in the other tissues, appears to be adequate to convert the usual amount of purine precursors into uric acid.

The effect of feeding p-dimethylaminoazobenzene on the xanthine oxidase activity of rat tissues is shown in Table II; uric acid and allantoin excretions are also given. The dye acted synergistically with a low protein diet in depleting the liver xanthine oxidase, and decreased the kidney enzyme independently of the protein content of the diet. The enzyme in lung, spleen, and intestine was not particularly influenced by the dye. The formation and excretion of uric acid and allantoin were normal on all the diets.

The loss of liver xanthine oxidase that occurs when rats are fed a purified diet is not

due to a "dilution" of the activity as a result of an accumulation of fat or glycogen. Three groups of adult Sprague-Dawley male rats were fed chow, a 21% or an 8% purified casein diet for 6 weeks. They were sacrificed under nembutal anaesthesia, and the livers were analyzed for xanthine oxidase, total fat by CHCl_3 extraction, and glycogen(6). The results, Table III, show that the liver xanthine oxidase can be decreased significantly by either purified diet without any significant change occurring in the liver glycogen or fat.

Summary. The depletion of liver xanthine oxidase by feeding adult or weanling rats a purified 8% casein diet did not interfere with the formation and excretion of normal amounts of uric acid and allantoin. Addition of p-dimethylaminoazobenzene to the diet was also without effect on this excretion. The loss of liver xanthine oxidase that occurred on a purified diet took place in the absence of any significant changes in fat or glycogen content of the organ.

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Influence of Lansing Virus Infection on Phosphorus Metabolism of Monkey Brain Tissue.* (17789)

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Recently there has been considerable interest in studies on the influence of viral growth on the metabolism of the host cell. Studies on the origin of phosphorus found in the bacteriophage virus particles(1,2), and studies on the influence of viral growth on the anaerobic glycolytic metabolic cycle of host cells(3,4,5), have been done using P^{32} as the tracer substance. Unfortunately, apart from the bacteriophage-bacterial cell studies, most of these investigations have been done *in vitro* using minced tissue preparations as culture media for murine strains of virus. As the transfer of phosphorus as phosphate ($-PO_4$) appears to be of great importance in the transfer of energy, both in the synthesis of nucleoprotein and in the metabolism of carbohydrate, it seemed advisable to study the metabolism of phosphorus in the intact animal during the development of the disease poliomyelitis. It was felt that determination of the amount of inorganic and total acid soluble organic phosphates in brain tissue and the rate of turnover of P^{32} into these phosphate fractions would provide information on the influence of a poliomyelitis virus on the metabolism of brain tissues.

Method. For these experiments, young conditioned healthy rhesus monkeys were given a single intracerebral inoculation of Lansing

poliomyelitis virus (Poliomyelitis Standard Commission). Both control and virus inoculated animals were then sacrificed at 12, 24, 48, 72, 96, 120, 144, 168, and 192 hours following inoculation. Fifty minutes before sacrificing, about one millicurie of P^{32} was injected intravenously in the form of an inorganic salt (NaH_2PO_4). Animals were sacrificed by bleeding from the neck. The brain, brain stem and cord were then removed rapidly and aseptically. Adjacent transverse slices from the medulla, pons, thalamus, spinal cord and cerebellum were obtained for (1) analysis of the content of acid soluble phosphate compounds, (2) determination of the specific activity of these phosphate compounds, (3) pathology, and (4) viral assay.

Results. These experiments demonstrate that associated with the development of pathologic changes and growth of the virus in the various nerve tissues studied, there were significant changes in the rate of turnover of phosphorus (P^{32}) in the inorganic phosphate fractions (IP) and in the total acid soluble organic phosphate fractions (TASOP). Table I presents the changes in P^{32} turnover rate of the various phosphate fractions in the virus inoculated animals as compared with the phosphate fractions obtained from tissues of control animals. The presence of virus, the presence of significant pathology and symptomatology are indicated in the table.

There appeared to be no significant change in the P^{32} turnover rate in the phosphate fractions obtained from any of the brain stem tissues of animals that were sacrificed at or before 72 hours following inoculation of the virus. At 96 hours, however, significant increases in the P^{32} turnover rate in the IP and the TASOP fractions were evident. These were first most striking in the medulla and in the pons tissues. Significant early pathology and evidence of viral growth were pres-

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TABLE I.
Change in the Rate of Phosphorus Uptake Into the Inorganic and Total Acid Soluble Organic Phosphate Fractions Prepared from Poliomyelitis (Lansing) Infected Monkey Tissues.

Time of sacrifice after virus inoc., hr	No. monkeys	% change in specific activity of phosphate fractions above controls										Pathology	Virus	Symptoms
		Medulla		Pons		Spinal cord		Cerebellum						
		IP	ASOP	IP	ASOP	IP	ASOP	IP	ASOP					
12, 24	4*	25	30	2	0	-30	-40	-20	32	0	T†	None		
48, 72	3	100	180	70	120	130	0	-40	-10	+	P, M	"		
96	3	420	350	250	260	140	160	60	80	+	P, M	Tremors		
120												Weakness		
192	3	196	195	220	115	426	403	160	156	+		Paralysis		

T, P, M = Virus present in thalamus, pons, or medulla.

IP = Inorganic phosphate.

TASOP = Total acid soluble organic phosphate.

* One monkey at each indicated time.

† Virus in thalamus only of 12 hr animal.

ent in these two tissues particularly by this time. At 120 hours after virus inoculation, when early symptomatology was manifesting itself, a most striking increase in the P^{32} turnover rate in the phosphate fractions obtained from the medulla, pons, and spinal cord was now observed. At this time, only tremors and slight weakness of the extremities was noted. By 192 hours, when advancing paralysis and increasing weakness were now evident, the changes in the phosphate fractions from the medulla and the pons seemed to decrease, but the fractions obtained from the spinal cord continued to show increases in P^{32} turnover rates. The fractions obtained from the cerebellar tissues now also showed significant increases.

A comparison of the P^{32} turnover rates in the phosphate fractions obtained from control and from experimental animals is justified on the basis of the fact that the diffusion curves of P^{32} from blood to spinal fluid was almost the same for control animals as for the inoculated animals. Further, the mean concentration of the specific activity in the blood and in the spinal fluid of control animals as compared with the blood and spinal fluid of inoculated animals was practically the same at the time of sacrifice. The relationship between blood and spinal fluid specific activity was 1:2.6 for control animals and 1:2.5 for the inoculated animals.

Discussion. It would appear from these studies that coincident with the early pathologic changes produced by a Lansing poliomyelitis virus in monkey nerve tissues, there is a marked increase in the turnover of P^{32} in the IP and the TASOP fractions of brain stem and spinal cord tissues. An increase in the rate of turnover of P^{32} in the IP fractions in these virus infected parts may be due to cell damage and associated changes in permeability of the tissues to phosphorus. However, the concomitant and equal increases in the P^{32} turnover in the TASOP fractions must be related to an increased metabolic activity within the cell. Whether this increased metabolism is due to an increased glycolytic metabolism or to increased protoplasmic synthesis (including the synthesis of the virus particles) remains to be studied.

It is of interest to note that the increase in metabolic activity was present first in the medulla, second in the pons, and third in the spinal cord. This sequence parallels the usual sequence of development of pathology and increased viral concentration in the intracerebrally inoculated animal.

Summary. Coincident with the development of viral growth and earliest pathology in the brain stem of Lansing poliomyelitis virus in-

oculated monkeys, there appears to be a marked increase in the metabolic activity of these tissues as regards the phosphate ion. This increase in metabolic activity of the affected brain stem tissues and spinal cord continues to increase during and throughout the period when clinical evidence of weakness, paralysis, and neurologic signs of clinical poliomyelitis exist.

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